IN VITRO STUDIES ON IMMUNOREGULATION

WITH SPECIAL REFERENCE TO RHEUMATOID ARTHRITIS

By

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M.B. Ch.B (Glasgow) M.R.C.P.(UK) FRCP(C)

Thesis submitted for the degree of

Doctor of Medicine

to

The University of Glasgow

From

The Rheumatic Disease Unit

University Hospital,

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SUMMARY

This thesis is based on studies conducted between 1979 and 1981 at the University of Western Ontario, London, Canada. Although considerable advance has been made in recent years in quantitation of various aspect of the immune response in rheumatoids, and in dissecting mechanisms by which the response is regulated in healthy individuals, there is a relative lack of information regarding immunoregulation in rheumatoid arthritis. A series of comparative studies were planned to examine quantitative and qualitative aspects of immunoregulation in patients with rheumatoid arthritis and normal healthy individuals.

The introductory chapters of the thesis review certain aspects of rheumatoid arthritis and immunology, immunoregulation in normal individuals and the immunopathogenesis of rheumatoid arthritis. Subsequent chapters report the collection, standardization, modification and application of study methods and discuss the results of serial studies.

For the functional studies, a modified reverse hemolytic plaque forming cell assay was used to measure immunoglobulin synthesis by B lymphocytes in cultures containing combinations of B and T lymphocytes. In this assay system responses (Studies 1-5) were shown to be related to culture duration, concentration and batch of pokeweed mitogen, source of foetal calf serum and the dose of irradiation used to manipulate the T cell help/suppression balance.

In Study 6 immunoregulation was examined by selectively destroying T suppressor cells with optimal dose radiation prior to coculture with B cells, and in Study 7 by selectively removing T suppressor cells using a chicken rosette assay which depleted T cell suspensions of cells bearing Fc receptors for IgG (Tγ cells) prior to coculture. In normal subjects, coculture of B cells with T cells enhanced the PFC (plaque forming cell) response, which was further increased when T cells were either irradiated at optimal dosages (T_{3200}) or depleted of Tγ cells (T nonγ) prior to coculture - a response consistent with an effective reduction of suppressor T cell activity. In marked contrast, while rheumatoid T cells were capable of enhancing the response of rheumatoid B cells to an equivalent degree as in controls, the additional augmentation seen when B cells were co-cultured with T_{3200} or T nonγ cells was absent in almost all rheumatoids. These observations were interpreted as indicating a functional and/or numerical deficiency in one or several subsets of mononuclear cells, but were not consistent with a pure dysfunction or reduction in the number of suppressor T cells alone. While abnormalities in T suppression in rheumatoid arthritis have recently been reported, the co-existence of other immunoregulatory abnormalities has not been examined. It was also demonstrated that the controversial Tγ cell population had suppressor activity

and that the radiosensitive suppressor cell and the Tγ cell were related. Finally, the restricted PFC response in rheumatoids was not found to be related to the number of Tγ cells, which was normal.

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Further evaluation of the functional status of rheumatoid lymphocytes (Study 11) demonstrated, that while rheumatoid B lymphocytes were capable of normally responding to the enhancing effects of allogeneic normal T_{3200} cells, that rheumatoid T_{3200} cells were incapable of normally enhancing PFC responses from

allogeneic normal B cells. These observations suggested, that RA B cells were normally responsive to helper signals but that RA T cell help was deficient. While monocyte function was not directly studied and required further evaluation, since RA monocytes predominated in the B cell fraction, it was inferred that RA monocyte function was likely normal.

In order to gain some appreciation of another aspect of cell communication, soluble factors were raised in 48 hour cultures (Study 12). Rheumatoid lymphocytes were shown capable of generating potent helper factors, which could effectively substitute for the presence of T lymphocytes in cultures, and which could cross allogeneic boundaries. While no significant difference could be detected between rheumatoids and normal controls, a more elaborate method was required before concluding that factor potency was normal. Alternatively communication by factors may have been normal but direct cell-cell communication may have been abnormal in rheumatoids, either as a consequence of an intrinsic abnormality of the cell or in its mechanism of direct communication.

Having observed normal RA B and inferred normal RA monocyte function, a final study was planned to confirm the impression that helper T cell function was abnormal. In Study 13, increasing numbers of T cells (non irradiated, T_{1250} and T_{3200}) were cocultured with B cells. Dose response curves differed between rheumatoids and controls. However, when a four fold excess of irradiated

T cells were cocultured with B cells, normal PFC responses were obtained in rheumatoids, suggesting that the restriction previously observed could be overcome by the addition of an excess of help. Finally, in normal subjects at low T/B ratios, low dose irradiation of T cells was noted to have a suppressor effect on the PFC response, confirming the presence of a radiosensitive population of helper T cells as reported by other researchers.

The studies reported in this thesis indicate that the dysfunction of suppressor T cells described by other investigators is not the only immunoregulatory abnormality present in rheumatoid arthritis, but that helper T cell function is also abnormal. The functional potential of B cells and monocytes has been considered normal although the latter requires further evaluation. Apart from an increase in the T4/T5 ratio in active rheumatoids, no quantitative abnormalities were noted in the numbers of immunocompetent cells involved in the abnormal responses observed. An association was confirmed between Tγ, OKT5+ and radiosensitive suppressor cells. In the final chapter, the relationship between these in vitro observations and the disease state are discussed and avenues for further investigation suggested.

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To

My wife, Helen and

my two children, Peter

and Catherine.

PREFACE

Before taking up the post of Research Fellow in Rheumatology at McMaster University, Hamilton, Ontario in July, 1981, my clinical training had been in internal medicine and rheumatology. My initial interest in the latter specialty developed during a junior house officer appointment with Professor E. McGirr at the Glasgow Royal Infirmary, part of which I spent with Professor W. W. Buchanan at The Centre for Rheumatic Diseases (1974). Having some appreciation of the clinical complexity of the specialty, I later went on to compliment this experience with appointments at Hammersmith Hospital, London under Dr. G.R.V. Hughes (1976) and in the University of Western Ontario Specialty Training Programme under Drs. Harth, Bell, McCain, Thompson, Disney and Coulter (1979-80). While these clinical encounters directed my interest towards the study of patients with rheumatic diseases, it was (in keeping with the history of immunology), Dr. Peter McKenzie who first raised my curiosity regarding the exact mechanism of the immune response and its regulation during my appointment as Senior House Officer in Infectious Diseases at Belvddere Hospital, Glasgow (1975). As an undergraduate, I had appreciated the differences which apparently existed between the humoral and cellular immune responses and committed Gell and Coombs classification to memory. However,

in between graduation fromGlasgow University in 1974 and undertaking the research to be reported in this thesis, major advances in the field of immunology had defined the existence of subpopulations of immunocompetent cells and generated several theories of how the immune response was regulated. While immune phenomena have been recognized for several decades in patients with rheumatoid arthritis and the consequences of chronic inflammation in this common disorder familiar to all practicing clinicians, the origin of such immune abnormalities has not been defined. A review of the literature revealed that while several immune abnormalities had been reported in rheumatoid arthritis, there existed new techniques which had not been applied in the study of the disease and which allowed examination of lymphocyte subpopulations and their regulation of the immune response. It was with enthusiasm that I accepted the opportunity to study immunoregulation in rheumatoid arthritis in collaboration with Dr. David A. Bell and Mrs. Ewa Cairns.

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ACKNOWLEDGEMENTS

I gratefully acknowledge the direction and generous support of my supervisor, Dr. D.A. Bell (Associate Professor of Medicine - University of Western Ontario), the technical assistance of Mrs. E. Cairns (Laboratory Technician) and the collaborative efforts of these two persons. The work was supported by a grant from the Canadian Arthritis Society. I am indebted to my colleaguesand patients in London, who participated in the studies. I am grateful to Mrs. M. French for typing manuscripts for publication and summaries for presentation in Boston and Toronto. In particular I acknowledge the dedication with which Mrs. P. Davis typed the entire thesis. A debt of gratitude is due to the members of the Department of Medical Illustration at McMaster University, Hamilton, Ontario, whose prompt and expert assistance facilitated the production of the final work. Finally, I acknowledge the generous support and encouragement afforded me by Professor W. Watson Buchanan, without whom this thesis could not have been completed.

The work contained in this thesis has been communicated to learned societies including the Inter-Urban Arthritis Society, American Rheumatism Association and the Royal College of Physicians and Surgeons of Canada. Some work is still in press, but some has

already been published:

Bellamy N., Cairns E. and Bell D.A. (1981).
Defective Immunoregulation in vitro in Rheumatoid Arthritis (RA).
Annals of the Royal College of Physicians and Surgeons of Canada, 14:240.

Bell D.A., Bellamy N. and Cairns E. (1981)
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Arthritis and Rheumatism, 24:S114.

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CHAPTER I

BACKGROUND TO RESEARCH (1)

- HISTORY OF RHEUMATOID ARTHRITIS
- AETIOLOGY OF RHEUMATOID ARTHRITIS
- HISTORY OF IMMUNOLOGY
- IMMUNOREGULATION AND THE IMMUNE RESPONSE

"Not to know what has
transpired in former
times is to continue
always a child".

Cicero. 106-43 B.C.

HISTORY OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a disorder unique to humans[1] although Bywaters[2] described a primate arthropathy with some clinical and histological similarity to the disease.

The antiquity of osteoarthritis has been clearly demonstrated in the mummified remains of the Pharoahs[3] and in the paleopathology of dinosaurs[4]. Furthermore, it can be recognized in mediaeval classical paintings and is eluded to in Shakespeare[5].

Although many have attempted to identify at what time in history rheumatoid arthritis first appeared, there is no definite description until the 19th century. Smith and Jones[6] described a prehistoric Egyptian skeleton showing fusion of one elbow joint suggestive of the disease, in a mummy found in a cemetery of the 5th Dynasty of Deshasheh in Egypt. The ancient Indian Sanskrit writings of Caraka Samhita (circa 100 A.D.)[8] may have alluded to the disease. Caughey[9] has provided good evidence that Emperor Constantine IX Monomachus (circa 980-1055) may have suffered from rheumatoid arthritis. It is not described in The Bible, the writings of Hippocrates and other ancient Greek medical

writers[10], Shakespeare[15] or any other text prior to the beginning of the last century. A review of mediaeval paintings[11] possibly provides some evidence for the presence of the disease in the Late Middle Ages. The metacarpophalangeal and proximal interphalangeal joint swelling of the right hand of the maidservant in Jacob Jorden's (1593-1678) The Painter's Family (Figure 1), and the swan-neck and buttonhole deformities seen in Jan vanEyck's (1441) Jan IV, Duke of Brabant bear striking similarity to those seen in rheumatoid arthritis.

It is controversial whether the English Physician, Thomas Sydenham[12] (Figure 2) was referring to rheumatoid arthritis when in 1685 he wrote -

> "Indeed it may happen that where the said pains will have harassed over many days and very often they may at length desist spontaneously, and meanwhile the sufferer may be deprived of all movements of his members until death, with the joints of the fingers as though reversed, and with swellings as in arthritis, knotted and protruding on the inside rather than on the dorsal part of the fingers; nevertheless he may have a good stomach and tolerate other aspects of life well".
>
> Sydenham 1685.

Figure 1 - THE MAIDSERVANT from JACOB JORDEN'S (1593-1678)
THE PAINTER'S FAMILY - possibly an early illustration of rheumatoid arthritis.

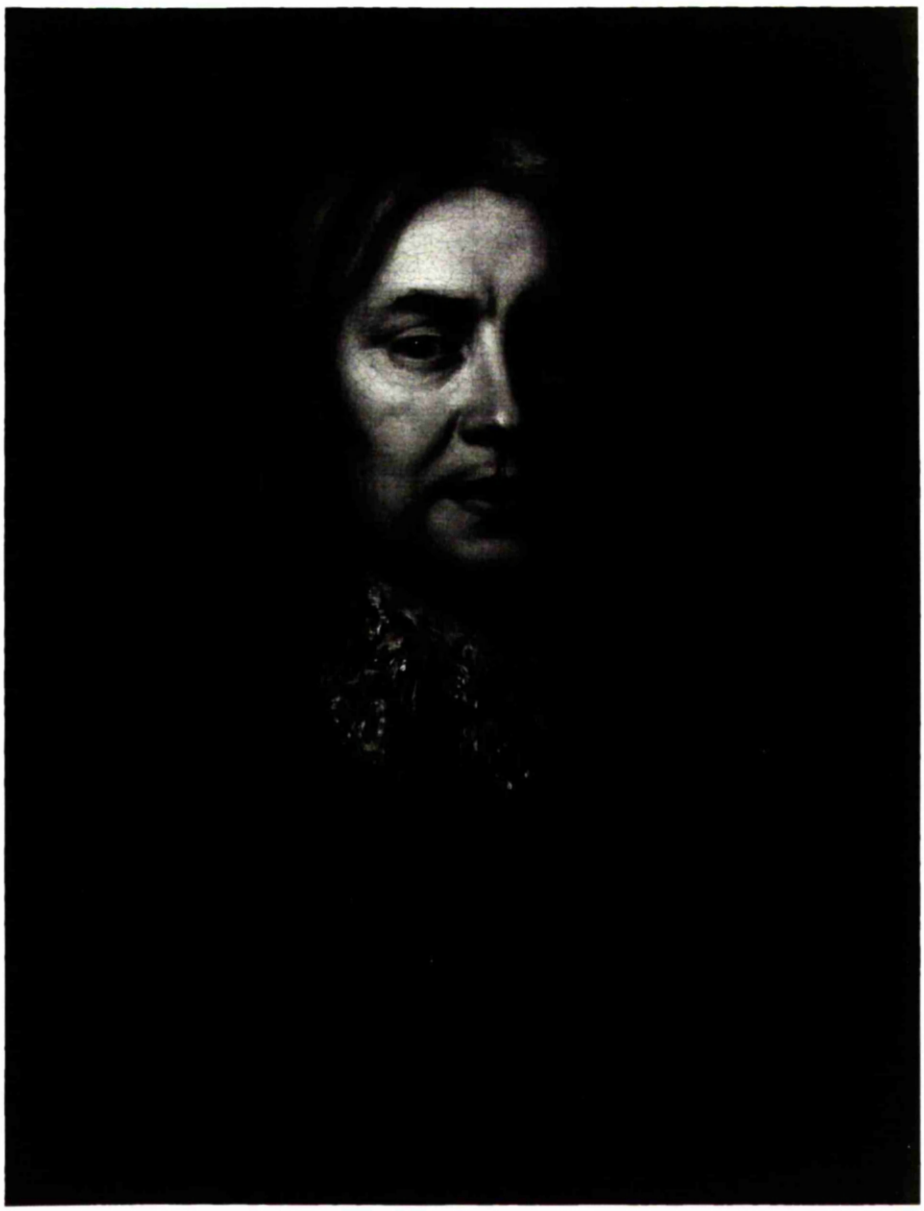


FIGURE 2 - THOMAS SYDENHAM (1624-1689)
"The English Hippocrates". From a portrait in the Royal College of Physicians, London.

The first classical description of rheumatoid arthritis is commonly attributed to Augustine Jacob Landre-Beauvais[13] (1772-1840). On August 3rd, 1800 at the University of Paris he presented a thesis entitled "Doit on admettre une nouvelle espece de goutte sous la denomination de goutte asthenique primitive".

> "a type of gout, less frequent indeed, but which depends on a condition of primary weakness. This is the disease of the indigent and has been rarely observed. One finds the common gout only among those who are able to live on a rich diet, then a condition of weakness follows a condition of strength and vigour. The 'primary aesthenic gout' has its seat in the houses of the poor".
>
> Landre Beauvais 1800
> (English Translation)

Sir Alfred Baring Garrod (1819-1907)[14] (Figure 3) used the name "rheumatoid arthritis" in 1859, although the terms rheumatism and arthritis have much older origin. Galen (131-201 A.D.)(Figure 4) introduced the term rheumatism from the Latin rheuma - a flux. The word flux was based on the humoral concept and signified that the disease arose from a flow of peccant humours. Of interest the Latin word gutta - a drop (origin of the word gout) similarly indicated a basis for disease arising from drops

Figure 3 - SIR ALFRED BARING GARROD (1819-1907)

First to use the term "rheumatoid arthritis"

Figure 4 - GALEN (131-201 A.D.) One time physician to the gladiators at Pergamum. Seen here performing the ancient art of "cupping".

of poisonous humours entering the joints and tissues. The term arthritis was used in the great writings of Hippocrates (Figure 5) to describe joint disorders.

There are a number of possible explanations for the apparently recent emergence of rheumatoid arthritis. (A) The disease was possibly mild or rare and escaped description by early authors and artists. (B) It could have been present for a long time but classified under a different name e.g. gout. (C) It may have been genuinely rare because in former centuries age expectation was lower and potential sufferers died of other causes before contracting the disease. The low prevalence of rheumatoid arthritis in Kenya has provided evidence of this explanation in under-developed countries[15]. (D) It could be, that it is indeed a relatively new disease to affect mankind. It has been postulated that rheumatoid arthritis is a viral disease. New viral diseases continue to appear e.g. English sweats of Tudor England, encephalitis lethargica, Bornholm disease, acute haemorrhagic conjunctivitis (Apollo disease), Marburg fever and Ebola viral haemorrhagic fever [16,17,18,19]. Could it be, that an as yet unrecognized virus, arising in the early 19th Century and persisting to the present time, is responsible for the

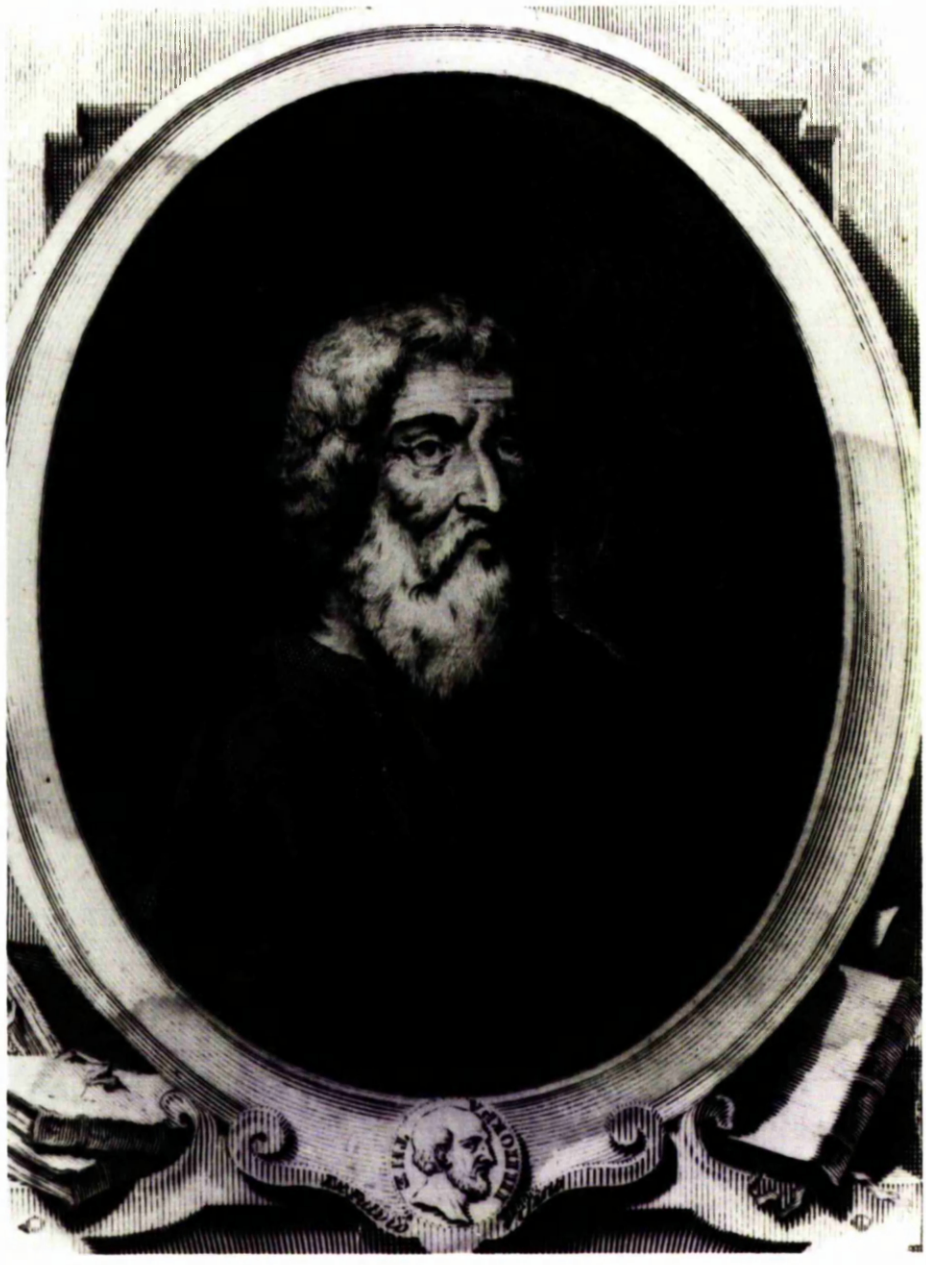


Figure 5 - HIPPOCRATES (460-370 B.C.)

In the 5th Century B.C. the lengthy development of Greek medicine was capped by and epitomised in Hippocrates.

disease? Or is the aetiologic agent non-viral, but similar in its evolution to that of Legionnaire's disease[20,21,22] - another apparently new condition? At present there are no answers to these questions.

The development of the nomenclature of rheumatoid arthritis spans several centuries[23]. Eventually recognized as a different disease to gout, ankylosing spondylitis, osteoarthritis and rheumatic fever, it was called by varying names - St. Gervasius' disease (collective term for any arthritis), rheumatick gout, primary asthenic gout and atrophic arthritis were some of them. The British Ministry of Health adopted the term rheumatoid arthritis in 1922, the American Rheumatism Association following suit in 1941.

Rheumatoid arthritis is a very common disorder affecting between 1 and 2% of the adult population of developed countries[24]. Apart from the islanders of Tristan da Cunha[25] no other community studied, has been found free of the disease. The relationship between cold damp climates and rheumatoid arthritis has attracted considerable attention. However, epidemiologic studies conducted on Black Feet Indians (from the cold semi-arid regions of Montana) and Pima Indians (from the hot, arid deserts of Arizona) have shown a

similar prevalence of rheumatoid arthritis in the two populations[26]. No relationship to latitude[24] has been discovered inspite of surveying Eskimos[27], European[28], Amerindian[26], Jamaican[24] and Bantu[29] populations.

AETIOLOGY OF RHEUMATOID ARTHRITIS.

The aetiology of rheumatoid arthritis is unknown but three hypotheses have attracted serious consideration - infection, heredity and auto immunity.

Before the technology needed to study auto-immunity was developed an infective aetiology was attractive. For several decades infective agents have been sought in the joints of sufferers from rheumatoid arthritis, but none has been demonstrated[30,31]. The varying enthusiasm for implicating particular classes of microbes has been generated principally by the available methodology: thus the sequence from bacteria to mycoplasmata and during the last decade to viruses.

Once favoured, diphtheroids have now been discredited as aetologic agents[32]. Serum levels of antibodies to enteric bacteria have not been found elevated in juvenile rheumatoid arthritis[33]. Although mycoplasma can be used to induce arthritis in laboratory animals, slow growing mycoplasma have only been found occasionally in patients with rheumatoid arthritis, the majority of patients showing no evidence of such an infection[34]. While a number of bacterial infections have on occasion arthritic manifestations, e.g. syphilis, tuberculosis, gonorrhea, and many

infections may stimulate the production of rheumatoid factor (although generally in lower titres than seen in rheumatoid arthritis) e.g. syphilis, leprosy, tuberculosis, schistosomiasis and subacute bacterial endocarditis, no agent has proven to be a consistent or likely candidate for an aetiological role.

Recently, interest in a viral agent has intensified. A variety of cellular inclusions have been noted but none convincingly viral in appearance[32]. Attempts at virus isolation have produced predominantly negative results. Immuno-fluorescent and radio-immunologic techniques have failed to identify virus particles[34,35]. Studies of virus antibody titres in rheumatoid arthritis have not identified a consistent abnormality, but have not usually included a control population. In the last few years the Epstein Barr virus (EBV) and two transformation antigens EBNA (Epstein Barr Nuclear Antigen) and RANA (Rheumatoid Arthritis Nuclear Antigen) have attracted considerable attention.

In 1973 Reedman and Klein[36] demonstrated that EBNA developed in B lymphocytes infected with EBV. Erberg et al[37] then showed a correlation between the presence of EBNA in infected cells and the amount of EBV DNA in the same cells. In precipitin and immunofluorescent studies

sera from patients with rheumatoid arthritis recognized this antigen. In vitro studies have shown that once EBV DNA is incorporated into B cell nuclei, it can persist for multiple generations and reproduce with each cell division, but does not usually progress to synthesis of the complete virion[38]. EBV stimulates the mitogenic activity of B cells and may lead to development of transformed cell lines that can in vitro be carried in perpetuity[39]. EBV is a potent polyclonal B cell activator of antibody production[40]. In one study[41], thirty per cent of the IgM antibody produced from infected rheumatoid B cells had high avidity rheumatoid factor activity, while from control cells only 2% had rheumatoid factor activity and it was of low avidity. Furthermore, rheumatoid cells produced much more rheumatoid factor. One possible interpretation of these differences was that rheumatoid lymphocytes were already maximally activated to rheumatoid factor production by the disease. B cell transformation was higher in rheumatoids than in normals and could be variably inhibited in vitro by the patient's T cells. This observation raised a question as to whether the problem was one of abnormally responsive EBV infected B cells, or of a reduced ability of T cells to suppress infected B cells.

Alspaugh et al[42] described the second transformation antigen, RANA which they felt differed antigenically from EBNA. Again in precipitin and immunofluorescent studies sera from patients with rheumatoid arthritis recognized the antigen. In one study of normal subjects[43] titres of EBNA and RANA in the same individuals were similar, indicating that they may co-exist.

In ascribing an aetiologic role to EBV there are a number of further considerations. (1) EBNA has been found in 75% of normals[43]. (2) EBV infects [illegible] of Americans by age 30 years[38], while the prevalence of R.A. is in the order of 1-2%. (3) There are a number of other microbial polyclonal B cell activators[38]. (4) T cell helper substances, macrophage stimulatory products, anti Ig antibodies, antibody to lymphocyte membrane antigens, and immune complexes reactive with Fc or complement receptors are all capable of provoking B cell mitogenesis[44,45,46,47]. While only EBV has been shown capable of provoking B lymphocytes to fully transform, these other factors may play a facilitatory role. In the light of this relatively new knowledge it is evident that EBV requires serious and further consideration as a major aetiologic agent.

If viral infection is important in rheumatoid arthritis, it likely results from the incorporation of the viral genome into the nucleic acid of host cells. There are a number of ways in which chronic synovitis could subsequently occur. (1) Neoantigens (either virus coded or virus-induced host-coded) might appear in or on the cell membrane of synovial cells and be susceptible to attack by the host immune system. (2) Virus coded membrane proteins may serve as antigens for immune complex formation. (3) Virus infection of lymphocytes and monocytes may impair immunologic functions and alter normal lymphocyte recirculation. (4) Virus transformed synovial cells may lose their normal control mechanisms, leading to chronic cellular dysfunction.

Heredity has long been considered an important factor in the aetiology of rheumatoid arthritis, but there is no evidence of a single dominant or recessive trait. O'Brien[48] invoked a mathematical model for estimating the probability of variable penetrance in chronic disease but failed to show any evidence for it in rheumatoid arthritis. In twin studies[49] the genetic component could only account for 30% of responsible aetiologic factors. However, seropositive erosive R.A. is 38 times more

likely to develop in an identical twin of a patient with this syndrome. The likelihood of seropositive erosive R.A. in a fraternal twin is that of a sibling of such a patient - sixfold increase.

More recently interest has centred on D locus genes of the HLA system located on the short arm of chromosome 6, and in particular on HLA DW4. DW4 occurs three to four times more frequently in adults with rheumatoid arthritis than in non-rheumatoid controls[50.51,52]. It is believed that D locus genes exert an influence on the immune response, and it is postulated therefore that genetic makeup may predispose some individuals to the development of rheumatoid arthritis. However, 39 to 45% of patients with R.A.[51,52] do not have DW4. Methods for accounting for these individuals [illegible] to suggest that genes presently recognized are in linkage dysequilibrium with those which really control the response, or that susceptibility is conferred at more than one gene locus. This may be true, but evidence is incomplete, and the aetiologic role of D locus genes cannot be estimated.

Immunologic tolerance - the mechanism that prevents auto-immune response, is poorly understood. Maintenance of tolerance appears to be the result of the combination of

(1) deletion or paralysis of autoreactive lymphocytes, (2) active inhibition of immune responses to self components by regulatory lymphocytes known as suppressor cells and (3) lymphocytes directed against specific antigen recognition units on the surface of lymphocytes, which inhibit antibody production and cellular immunity. It appears that autoimmune phenomena can be initiated by exposure of the immune system to host components that are normally inaccessible, or by alteration of self components evoking cross reactivity, or from malfunction of the normal regulation of the immune system. The interaction of viruses or chemicals with self components in plasma membranes, particularly those coded for in the major histocompatability complex, may be critical in initiating certain disease states.

The concept of autoimmunity in rheumatoid arthritis originated from the observation that the serum of rheumatoids would agglutinate many organisms, and even inert particles[53,54,55]. It was soon shown that this phenomenon was due to the presence of rheumatoid factor which acted as an antibody to gamma globulins, and thus was considered an auto-antibody[56]. Different classes of rheumatoid factor are now recognized, producing some difficulty with the terminology, seropositive and seronegative. Patients who are seronegative for IgM rheumatoid factor may be positive for IgG rheumatoid factor.

The pathogenetic significance of rheumatoid factor has to be viewed in the light of several observations. It is evident that IgM rheumatoid factor is not essential for the existence of the disorder since approximately 20% of patients have no detectable IgM RF[57]. Furthermore, rheumatoid factor may be found in healthy normal subjects[58] does not harm normal volunteers when administered intravenously[59], and one third of children with agammaglobulinaemia develop a disease which has all the features of rheumatoid arthritis without demonstrable rheumatoid factor being present in their blood[60].

Evidence implicating rheumatoid factor in a pathogenic role will be discussed in the section on immunopathogenesis of rheumatoid arthritis. The demonstration of antibodies to IgG, nucleoprotein and collagen in serum and synovial fluids of patients with rheumatoid arthritis suggests an impairment of discrimination between self and non-self. How many of these phenomena are primary and reflect causation, and how many are secondary and are a consequence of the disease is not known.

The pathology of rheumatoid arthritis is primarily that of severe chronic inflammation. Although this occurs mainly in the synovial lining of diarthrodial joints, no tissue is exempt from the ravages of the disease. There is evidence implicating both cellular and humoral immune responses. Large numbers of T lymphocytes in synovial tissue and lymphokines in synovial fluid, suggest a role for cell mediated immunity[34]. Rheumatoid synovium in vitro produces antibody, and this together with the identification of immune complexes in synovial fluid assert the importance of humoral immune mechanisms.

Thus, it is probable that rheumatoid arthritis is of multifactorial aetiology rather than due to a single cause. Genetic effects are likely mediated through an influence on the immune response, resulting in increased host susceptibility, while the initiating event for the disease may be a slow virus. To date no relationship has been established between DW4 and anti-RANA and anti-RDNA[43]. However, there are undoubtedly other D locus antigens which will be defined in the future, and there are in addition several naturally occurring polyclonal B cell stimulators other than Epstein Barr virus[38].

HISTORY OF IMMUNOLOGY

Thucydides at the height of a plague in ancient Athens recorded that the sick and dying would have received no nursing at all, had it not been for the devotion of those who had already had the plague and recovered from it, since it was known that no one ever caught it a second time[61]. From at least 1500, the Chinese and Turks had both practised prophylactic induction of smallpox in their children. In China this was achieved by taking dried powders of smallpox crusts in the form of snuff - a practice not without occasional misfortune. Lady Wortley Montague (in the reign of George I) was the first Briton to report the benefits of this form of treatment, which inspite of religious objections she allowed to be successfully administered to her child in Constantinople. The germ of immunology grew out of the common knowledge that those who survived an infectious illness seldom contracted the disease again during their lifetime. Anthony von Leeuwenhoek (1632-1723) (Figure 6) made monumental contributions to medical science by introducing (but not inventing) the microscope, by the description of his "little animals" and by his recognition of the corpuscular elements of human blood. With the availability of the microscope and the knowledge of the existence of micro-organisms Edward Jenner (1749-1823) ensured the future of

Figure 6 - ANTHONY von LEEUWENHOEK (1623-1723)

Introducer of the microscope to medicine and describer of "little animals".

immunobiology when, as a medical student, he discovered that innoculation with cowpox crusts, protected humans from smallpox. He made this observation in milkmaids who had contracted cowpox and were subsequently resistant to smallpox. Louis Pasteur (1822-1895) first used the word vaccine (vacca:a cow - in honour of Jenner). He developed the germ theory of disease, refined preventative immunization, and was able to use living attenuated cultures of cholera organisms to actively immunize fowl. Pari passu with the development of knowledge of the important part that bacteria played in the causation of human disease, interest increased in the process by which the body resisted invasion by micro-organisms and by which immunity was achieved following infection[62]. Pasteur himself was well aware of many of the problems and thought vaguely in terms of a struggle between host cells and invading organisms. Robert Koch (1843-1910) discovered the tubercle bacillus, and in his attempts to develop a vaccine for tuberculosis, observed the phenomenon of delayed hypersensitivity (cell mediated immunity)[63].

It was not until 1883 that the Russian Zoologist Elie Metchnikoff (1845-1916) (Figure 7) realized the potential importance of a cell which fought infection.

Figure 7 - ELIE METCHNIKOFF (1845-1916)

Leader of the cellular school
of immunology.

> "One day I remained alone with my microscope, observing the life in the mobile cells of a transparent starfish larva, when a new thought suddenly flashed across my brain. It struck me that similar cells might serve in the defence of the organism against intruders ..... if my suspicion was true, a splinter introduced into the body of the starfish larva, devoid of blood vessels or a nervous system, should soon be surrounded by mobile cells as is to be observed in a man who runs a splinter into his finger .... that experiment formed the basis of phagocyte theory, to the development of which I devoted the next twenty-five years of my life."
>
> E. Metchnikoff 1883.

Although Neisser, Koch and Ogston had recognized that bacteria were often found within blood leucocytes, they had failed to appreciate the significance of their observation and assumed that leucocytes had been invaded by bacteria. In fact Panum, Ewart and Shafer had also recognized phagocytosis but did not attribute to it, its due importance and therefore failed to pursue the idea. By 1891 Mechnikoff had traced the evolution of the reaction of living organisms to parasitic invasion or injury, and in so doing put his finger on the significance of inflammation - it was a reaction for the defence and repair of damaged tissue carried out by phagocytic cells. For several years he worked with the

transparent waterflea (Daphnia), an ideal model in which to microscopically observe in vivo the activity of blood corpuscles. In his later life he recognized two main phagocytic blood cells, polymorpho-nuclear leucocytes and monocytes, and also recognized tissue macrophages. Metchnikoff's theory of phagocytosis was not generally accepted at the time, although Pasteur found it plausible (a fact which resulted in Metchnikoff moving to the Pasteur Institute in Paris).

The next major advance was the development of the theory of humoral immunity[62]. It was Nuttall who first recognized that defibrinated blood was capable of reducing the number of viable organisms on culture plates. Two of Koch's assistants, Behring and Nissen, extended this work and demonstrated that blood was only bacteriocidal against some organisms. Then Buchner in 1889 showed that this bacteriocidal property was present in "cell-free blood sera", a fact which provided the impetus for a school of thought differing greatly in its philosophy from that of Metchnikoff's. Four years later Buchner discovered complement (alexine).

Roux and Yesin (1885) working with diphtheria bacilli demonstrated that the organism elaborated a potent soluble exotoxin, an observation now known to be of immense pathogenic importance. Behring's major contribution (for which he became the first Nobel Lauriate in medicine and physiology) was the recognition of anti-toxin. It was discovered that guinea-pigs could be immunized against diphtheria toxin and that sublethal doses of diphtheria resulted in the production of an animal immune to the disease. Behring conducted similar experiments on tetanus with Kitasato, and it was not long before antitoxins were produced commercially.

At the Congress of Hygeine in London in 1891 the humoral theory was favoured[62]. Following heated debate at this Congress Behring and his colleagues in Koch's laboratory went on to further discovery. While Lister kept an open scientific mind about the two philosophies (though favouring the cellular school), Metchnikoff sensed that his credibility was being challenged, and rather than attempting to reconcile the two, persisted in attempts to demonstrate the superiority of the cellular school and made no further contribution of any significance to the science of immunology.

The closing years of the 19th Century saw intensive work on various bacteria and the recognition that appropriate immune sera could immobilise vibrio cholera and agglutinate typhoid bacteria. Pfeiffer, Gruber, Widal and Wright made their individual contributions around this time. However, Border made the most important observation, that serum from one species would agglutinate the erythrocytes of another[62]. Italian workers showed that the serum of a horse injected with rabbit erythrocytes, acquired toxic properties for the rabbit. Furthermore, immunization of animals with bacteria, enhanced the power of their serum to agglutinate the same bacteria. It was therefore considered and subsequently proven that the same process would increase the agglutination power of serum against foreign erythrocytes. Bordet demonstrated that agglutination and lysis required both heat stable antibody and heat stable complement.

At the turn of the Century Metchnikoff led the cellular school and Ehrlich had risen to prominence as the leader of the humoral school. Paul Ehrlich (1854-1915) (Figure 8) has been considered one of the greatest scientific workers in basic science. In three years he founded morphological haematology (normal and pathological) and recognized subsets

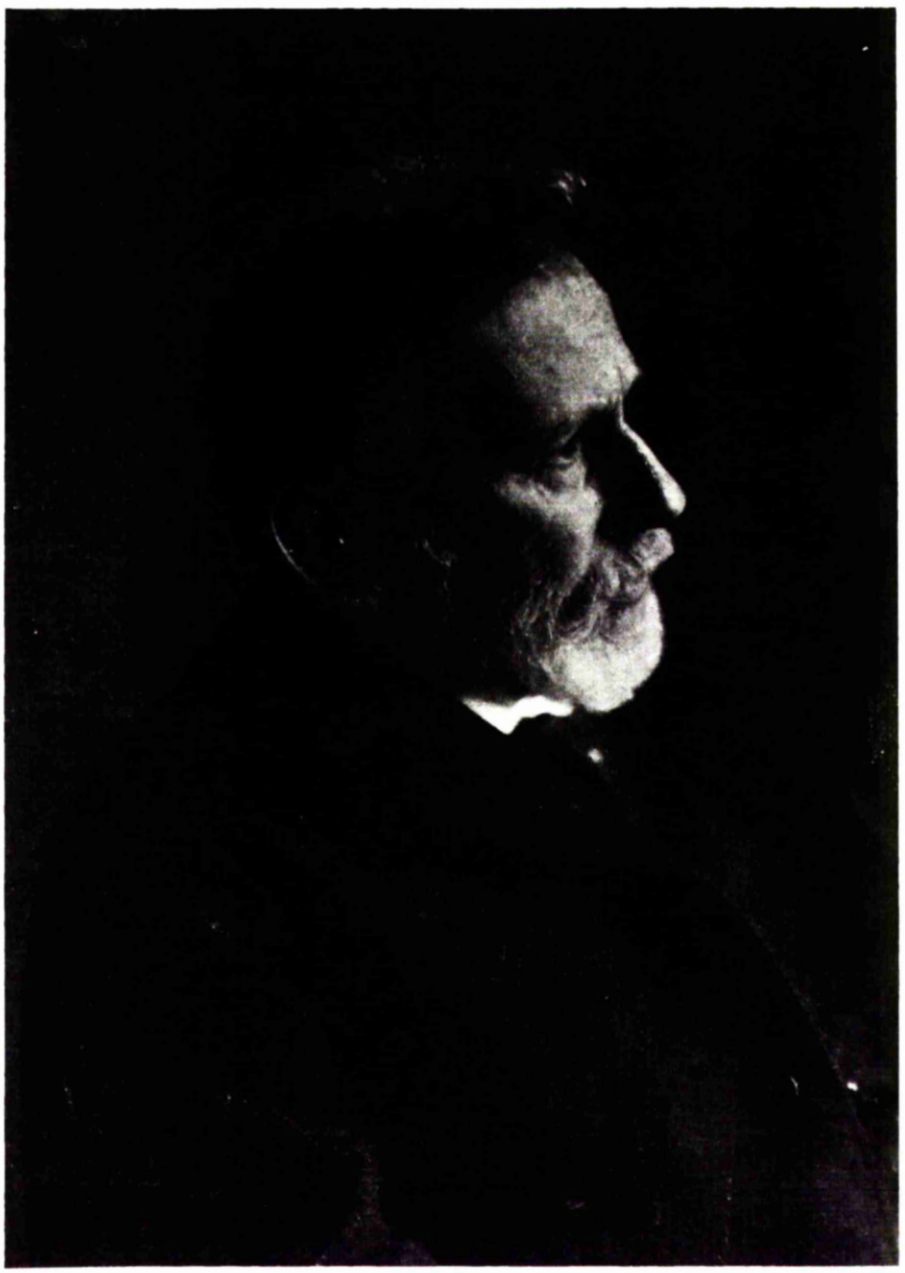


Figure 8 - PAUL EHRLICH (1854-1915)
Leader of the humoral school
of immunology.

of white blood cells by their staining characteristics. Ehrlich made remarkable discoveries in several areas of basic science. His contribution to immunology can be divided into five periods[62].

1890-1893: Studies on immunity to vegetable poisons ricin and abrin and connected studies on the inheritance of immunity.

1894-1898: Work on production and assay of potential diphtheria antitoxin.

1899-1901: Studies on immune haemolysis (prompted by Bordat's observations).

1901-1903: Evolution of a general theory of immunology, attempting to explain and reconcile previous observations.

1904-1906: Attempts to fit new facts into the theory and encouraging the activities of his pupils.

From his work on tetanus antitoxin Ehrlich developed the so called sidechain theory[63], in which it was conceptualized that sidechains anchored large molecules to cell surfaces (bacteria or red cells), while at the same time

coupling the complement necessary for cell lysis. Furthermore, excess sidechains were released to circulate free as an antitoxin in the blood. The next few years saw intensive work on haemolysins, antitoxins and the nature of complement and red cell receptors. Ehrlich recognized the possibility of horror autotoxicus[64] (auto-immunity) but failed to recognize it clinically in humans or in animals. Ehrlich's sidechain theory was not universally accepted and was seriously challenged by Bordet, Arrhenius and Gruber. Amongst his other discoveries were the lag time between antigen injection and antibody production, the anamnestic response, the central transfer of immunity, and the renaming of Bordet's alexine, complement. It was fitting that in 1908 he shared the Nobel Prize with Metchnikoff.

In 1903 Almoth Wright and Stewart Douglas recognized that neither the cellular theory nor the humoral theory was wrong and that both were correct[65]. By coining and defining the term "opsonin" the differences between the two schools of immunology were resolved. Two French scientists Portier and Richet (1902), working with sea anemone toxin in dogs described anaphylaxin and for the first time the detrimental

effect of antibodies was recognized[66]. Since this was the era of antiserum injection it had a profound impact on the practice of medicine. In the years between 1900 and 1930 Schick and vonPirquet described the serum sickness reaction (1905), Prausnitz and Kustner recognized reagin (IgE) (1921), Landsteiner discovered the ABO blood group system (1903) and coined the term hapten (1917), while Wasserman developed a test for syphilis (1906).

The modern era of immunology[65] began in 1930 when Haurowitz formulated the template theory of antibody formation, which suggested that antibody forming cells retained the antigen, and used it as a template for antibody production. As a consequence antigen and antibody had physical compatibility. Burnet's theories of antibody formation evolved slowly and in 1954 he conceived the clonal selection theory[67]. His theory differed from that of Haurowitz, in that it stated that clones of cells were genetically capable of making a certain antibody and that antigen recognition caused these cells to proliferate and produce specific antibody. Burnet also suggested that self recognition occurred in neonatal life by contact of antibody forming cells and a newly formed foetal antigen. This resulted in tolerance by suppression of forbidden clones. For this work on immunologic tolerance in conjunction with Medawar he received the Nobel Prize in Medicine and Physiology in 1960.

In the last thirty years groups of research workers have developed new methods for studying the immune response[65]. Ouchterlony, Oudin and Elek (1946-1948) developed gel diffusion tests, Gruber and Williams (1953) immuno-electrophoresis and Berson and Yalow developed radio-immune assay procedures (1959). Kabat and Tiselius recognized that antibodies were globulins (1938) and Porter (1958) and Edelman (1959) elucidated their structure. In 1957 Alick Isaacs (1957) discovered Interferon, an antimicrobial agent lacking the specificity of antibody. In 1956 Ernest Witebsky demonstrated the aut-immune nature of Hashimoto's thyroiditis and opened a new era of medicine.

In the last twenty years the understanding of the complexity of the immune response has progressively advanced[65]. Gell and Coombs classified the immune response into four types[68]. Type I reaction (anaphylactic, ragin dependent, release of vascoactive hormones): Type II reaction (cytotoxic): Type III reaction (Arthus-type, damage by toxic complexes) and Type IV reaction (delayed, cell-mediated). The heterogeneity of circulating lymphocytes and their recirculation characteristics were described by Gowans[69] (1966). Since then the study of cells and tissues of the lymphoid system has been prolific. Although Glick[70] accidentally discovered the importance of the bursa of Fabricius in antibody production, it was Miller[71] in 1961 who noted the

role of the thymus in immunologic development. In the 70's it was discovered that B and T lymphocytes could be differentiated by surface characteristics[72]. A role for the monocyte, separate from that of pure phagocytosis, was recognized and an intensive investigation begun into the interaction which occurred between different subsets of mononuclear cells in the immune response[73]. In the last ten years the importance of the immune response genes of the HLA system has been realized[74]. In the 1980's it can be anticipated that substantial progress will be made in immunogenetics, our comprehension of the regulation of the immune response and in the development of new methods for studying both.

IMMUNOREGULATION

AND

THE IMMUNE RESPONSE

The lymphoid system has three components[75]: (1) stem cell pool, (2) the primary lymphoid organs (thymus and bursa of Fabricius) and (3) the secondary lymphoid organs (lymph nodes, spleen lymphoid aggregates). Within this system there are at least four important cell populations: B lymphocytes, T lymphocytes, null cells and monocyte-macrophages. The expression of the immune response relies not only on these cells but also on accessory cells (e.g. granulocytes), and biologically active proteins (e.g. complement).

Stem cells are pluripotential cells having the capacity to replicate, self renew and differentiate into more mature forms. Originating in the yolk sac and liver prior to birth they reside in the bone marrow in the adult. These cells are eventually released into the blood stream and further differentiate, depending on the micro-environmental factors.

The thymus and bursa-equivalent lymphoid organs are important in this further differentiation, and confer on these immature cells those features which result in the production of T (thymus-dependent) or B (bursa dependent) lymphocytes. Once released from these organs it is believed that both T and B cells recirculate from the blood through lymphoid tissue, enter lymph nodes and return to the blood via lymphatic ducts. Moreover, it is

thought that B cells and T cells populate different areas of lymph organs. Thus, removal of primary lymphoid organs prior to the differentiation of T and B cells, impairs the capacity of an individual to undertake certain immune responses[76]. Thymectomy impairs cellular immunity and whilst not influencing the generation of immunoglobulin producing cells, does impair some types of immune response dependent on T/B cell interaction. Removal of primary lymphoid organs in the adult does not affect the response unless the pool of immunocompetent cells is also depleted.

Differing biological properties and functions of B and T lymphocytes, null cells and monocytes have been recognized in recent years.

B Lymphocytes:

Precursors of antibody secreting cells are recognized by the presence of intrinsic immunoglobulin on their cell membrane (mIg). No other immunocompetent cells bear immunoglobulin. It is not detectable on T cells, while the passively acquired Ig on monocytes and null cells can be readily dissociated from the cell membrane. These cells can thus be identified using an anti-immunoglobulin bearing a detectable marker e.g. fluorescein or

rhodamine. Two sources of error require identification. Firstly, many cells (including B cells) have receptors for Fc regions of aggregated immunoglobulin[77] and therefore cells may bind aggregates of labeled anti-immunoglobulin antibodies through reaction of their Fc receptors with Fc portions of the antibody, and be identified as bearing mIg, when in fact they lack mIg but possess Fc receptors. This may be eliminated by removal of aggregates from a preparation of labelled anti-Ig immediately prior to use. Secondly, Fc receptors (on non-B cells) may already have bound immunoglobulin in vivo and thus appear to possess mIg[78]. This effect can be reduced by incubating the cells at 37°C and washing them prior to examination - a procedure which dissociates passively acquired immunoglobulin.

As well as possessing mIg and Fc receptors, B cells display cytoplasmic immunoglobulin (cIg), receptors for C_3b and C_3d and Ia antigens.

Knowledge of the ontogeny of human B cells is incomplete. It appears that the first human mIg^+ cells are detected at 10 weeks of gestation. These cells have IgM on their surface but lack complement receptors for Ia antigens. With maturation, additional membrane markers appear i.e. IgD, Ia antigens and

complement receptors. Finally, the class of immunoglobulin which that cell is programmed to secrete is expressed. The switch in immunoglobulin class expressed is not accompanied by a change in the specificity of the immunoglobulin of that cell. Since IgD is found only in low serum concentrations in humans, it seems likely that its principle role is as a membrane receptor and not that of a secreted antibody.

The initial event in cell activation is that of antigen binding to a membrane receptor. These receptors have been demonstrated to be mIg molecules[79]. It has been estimated that $5x10^4$ to $1x10^5$ mIg molecules (of IgD or monomeric IgM) exist on the B cell surface. Membrane immunoglobulin is orientated such that its Fc receptor portion and its antigen binding site are exteriorized. mIg is mobile within the plane of the membrane and can migrate to form micro-aggregates (patches) or clumps (caps) in response to multivalent antigens and anti-Ig antibody[80]. Furthermore, it has been noted that following cap formation there is endocytosis, which results in an initial loss of membrane receptors followed by synthesis of new mIg. This relationship between mIg and cytoskeletal structures may relate to the signal generation necessary for lymphocyte activation[81].

Burnets clonal selection theory suggested the existence of a heterogenous group of lymphocytes each capable of synthesizing antibody to only one type of antigen. An antigen was visualized as coupling to one of these cells, causing proliferation and differentiation to produce a clone of cells with the same specificity as the parent cell. Subsequent studies using radiolabelled antigens have supported this theory, in that only a very small percentage of B cells bind the antigen. Furthermore, anti-immunoglobulin prevents binding of the antigen to the mIg receptor[82]. The second requirement of Burnets theory was that the specificity of the cell receptor must be the same as that of the secreted product of the descendants of that cell. Evidence to date supports Burnets original theory[83,84].

Antigen binding does not necessarily result in activation and therefore other factors must be involved. The mechanics by which B cells are activated are poorly understood at present[85]. There are two favoured mechanisms (a) antigen initiated changes in membrane associated adenyl cyclase, producing changes in the levels of cyclic mucleotides; (b) local aggregation of receptor molecules resulting in a flux of Ca^{++} ions through the formation of membrane channels. It should be noted, however, that results have not been reproducable between laboratories and

the use of polyclonal activators may not equate with the mode of activation encountered in vivo to specific antigens. Nevertheless, pokeweed mitogen has proven a particularly valuable tool in the study of human B cell function.

T-lymphocytes:

The thymus-dependent (T) lymphocytes have two types of activity - regulatory and effector.

Regulatory:
(1) Helper function - T cells help B cells respond to T dependent antigen.
(2) Amplifier function - T cells amplify certain T mediated effector functions.
(3) Suppressor - T cells suppress antibody synthesis and some T effector functions.

Effector:
(1) Cell mediated cytotoxicity - these cells specifically kill antigen bearing cells.
(2) Lymphokine production - activated lymphocytes release potent nonspecific biologically active substances having effects on (a) macrophage activation (b) chemotaxis (c) target cell lysis (d) resistance to viruses.

Histologically indistinguishable from B cells with the light microscope, T cells are recognized by their ability to form rosettes with sheep erythrocytes[86]. That is for sheep red cells to cluster around a central human T lymphocyte. These cells also possess Fc receptors, some having receptors for aggregated IgM

(Tμ cells) and some for aggregated IgG (Tγ cells). Available evidence suggests that these receptors may be markers for distinctive functional subsets of cells[87]. The recent development of monoclonal anti-T cell antibodies produced by the hybridoma technique affords a method for identifying and enumerating T cells by a different method[88]. The ontogeny and development of T lymphocytes has attracted considerable attention. Immature T cells migrate to the thymus where they further differentiate and acquire specificity. (Much of this early work was conducted in mice and the principles extrapolated to humans). Entering at the periphery of the thymic cortex, lymphocytes (thymocytes) vigorously proliferate and begin their migration towards the medulla. It appears likely that thymopoeitin exerts a significant effect on maturation. Immune competency is conferred within the thymus possibly through the interaction of thymocytes with self antigens expressed on thymic epithelial cells and/or thymic macrophages[89]. The high density of macrophages at the thymic corticomedullary junction has yet to be adequately explained, but may be an important factor in the production of immune competence. The mature lymphocyte possessing surface receptors and immunologic competence migrates to the circulation and then to peripheral lymphoid tissue.

T-helper function:

Helper function was first recognized by the study of antibody responses to sheep erythrocytes[90]. When irradiated mice were reconstituted with bone marrow cells only, or thymocytes only, they failed to produce antibody on immunization with sheep red blood cells. However, if they received both marrow and thymic cells they made an excellent response. It has been demonstrated in a mouse model that the helper T lymphocyte is antigen-specific and exerts its helper function only if stimulated by the antigen to which it is responsive[91]. Since the helper effect is most efficient when collaborating B and T cells are brought into intimate contact (as it is when each reacts with distinct determinants), it suggests that help is mediated either by direct cell-cell contact or via a soluble factor with a short range of action. In the last few years it has been recognized that culuring primed T lymphocytes with antigen leads to the secretion into the medium of a factor which specifically helps the response of B lymphocytes to antigens on the same molecule for which the T cell was specific[92]. It appears that these factors are not conventional immunoglobulin molecules, although they possess an antigen binding site.

T-suppressor function:

Recognition of the modulating effect of T cells on antibody production has been most important[93]. Suppression has been demonstrated to be antigen specific and can be mediated by soluble factors in mice[94]. Thus helper and suppressor factors are similar in that they are both T cell products, specific for antigen, lack antigenic determinants for immunoglobulin but possess antigenic determinants coded in the MHC. They differ, in that (1) helper factor is secreted into the culture medium, while suppressor factor is obtained by disrupting cells and (2) I antigens are coded for by different I subregions of the MHC (help I-A, suppression I-J mice).

The site of action of the T suppressor cell is unknown. It could act directly on the B cell and suppress activation, or it could act on the T helper cell and indirectly decrease B cell activation. Evidence presently favours the latter. Suppressor cells which exert potent nonspecific effects on antibody formation have been described and may be important in the normal regulation of the immune response. Concanavallin A treated T cells and the soluble factor SIRS (soluble immune response suppressor), have been shown to suppress a wide range of immune responses in a nonspecific fashion[95].

T-amplifier function:

Killer T lymphocytes may be primed in vitro by culturing them with potential target cells. Priming is much more efficient however, in the presence of a second population of T lymphocytes (amplifier cells) which are also specific for antigenic determinants on the target cell.

Cytotoxic T cells:

The killer T cell is important in cell mediated destruction of allogeneic cells and of cells bearing virus and tumour antigens[96]. It appears that a single T cell can destroy multiple targets and that the interaction time necessary for cytotoxicity is short. Killer T cells can act in isolation of other cell types, although the latter have a profound effect on the development of K cells as noted above.

Lymphokine production:

Lymphokines are a group of biologically active substances amongst which are -

Macrophage Migration Inhibition Factor (MMIF)

Macrophage Activating Factor (MAF)

Chemotactic Factors for monocytes, granulocytes and eosinophils.

Interferon

Products toxic to tumour cells.

In general lymphokine production depends on T cell activation, although B cells may initiate it or even secrete lymphokines themselves (as may monocytes). These products have a profound effect on the inflammatory response, tumour cells and viruses.

Evidence suggests that T cells utilize a different antigen recognition system from B cells. Although knowledge is still incomplete it appears that T cell activation requires the recognition of both a foreign antigen (non-self) and of an I region antigen common to the antigen presenting cell and the primed T cell (self)[97,98]. This requirement has been termed the "MHC restriction of T lymphocyte activation" and suggests a major function for the I region of the MHC. This point has been further highlighted by the observation that antisera directed against I region gene products inhibits T lymphocyte activation[99]. Although the nature of the receptor is unknown, MHC gene products and Ig V_H gene pool products (genes coding for idiotypes on the variable region of the heavy chains of antibodies) are strong contenders.

Monocytes and Macrophages:

The monocyte-macrophage system has gained increasing importance in the initiation and mediation of the immune response[100]. Monocytes derived from the haemopoietic system migrate into peripheral tissues where they are identifiable as macrophages. These cells have some similarity to both polymorphonuclearleucocytes and lymphocytes, in that while being phagocytic cells they also bear receptors for IgG and C_3 and in some cases Ia antigens. The functions of monocytes are many and varied -

(1) Phagocytosis
(2) Antigen processing and presentation
(3) Release of biologically active products.
(4) Tumour surveillance.
(5) Angiogenesis
(6) T lymphocyte differentiation.

In the present context, antigen recognition and subsequent presentation are the most important functions since they initiate the immune response. Antigen binding may occur through several mechanisms which may be either specific or nonspecific. Thus it has been observed that when antigen is incubated with monocytes it tends to stick to the cell membrane. The molecular basis of

this process is unknown. However, Fc receptors on the monocyte cell membrane act in a more specific fashion and may bind indirectly to antigen, either by prior attachment of cytophilic antibody (which then binds antigen) or by binding antigen/antibody complexes. Cytophilic antibody binding to antigen is likely important at low concentrations of antigen since at high concentrations its avidity is exceeded by nonspecific binding. Finally, monocytes can bind complexes through their C_3 receptors.

As previously described antigen presentation by a syngeneic adherent cell is critical to activation of most T cell functions. Whether the monocyte is invariably the cell which presents the antigen is not known. While there is evidence to suggest that the act of presentation is passive, in that antigen is simply displayed on the surface of an Ia^+ monocyte, it has also been demonstrated that an active process requiring antigen processing results in the creation of an immunogenic moiety [101,102]. Thus the nature of antigen presentation is unclear. However, Rosenthal's guinea pig experiments have illustrated the key role that monocytes play in directing the immune response[103]. The way in which the monocyte activates the T cell has not been identified, it may be by cell-cell interaction at the time of antigen presentation, although a lymphocyte activating factor (LAF) has been described.

LAF potentiates the response of both B and T lymphocytes and may act on a cell which has bound antigen on a monocyte surface.

The elegant work with Listeria monocytogenes infection in mice by Mackanass and Blanden[104] drew attention to the critical role played by these cells in the effector sequence. It was determined that Listeria immune T lymphocytes cocultured with macrophages from a nonimmune donor could heighten the in vitro bacteriocidal actions of the macrophages when Listeria were added to the cultures. This enhancement was dependant on T cell lymphokines, particularly macrophage activating factor, a process which can be mimicked by adding suitable lymphocyte culture supernatants to macrophage cultures.

Null Cells:

These cells cannot be characterized as B cells, T cells or monocytes and hence are termed null cells. It is not known whether these cells are homogenous or whether they are precursors or unrecognizable forms (by current techniques) of T or B cells. Since they are found in humans with agammaglobulinaemia and with T cell deficiency it is likely that they are a distinct cell

line. Two important functions have been ascribed to these cells which constitute ten per cent of the human peripheral blood lymphocyte population. (1) Antibody dependent cell mediated cytotoxicity (ADCC) (2) Natural killer (NK) activity. In the ADCC response null cells are capable of destruction of antibody coated nucleated target cells - a process independent of that produced by complement or killer cells. (The null cell binds antibody by its Fc receptor) Natural killer activity refers to the ability of null cells to destroy targets not sensitized by antibody. This area is one of significant controversy and intense interest at the present time.

An appreciation of the complexity of immunoregulation requires integration of the principle elements of the immune response which have already been eluded to. Thus in a normal immune response evidence indicates that antigen is bound to the surface of a monocyte and then presented to a T lymphocyte. Interaction requires the recognition of I region gene products and antigen on the cell surface of the monocyte by the syngeneic T cell. This interaction results in T cell activation and the release of lymphokines and helper factors. (It has been suggested that free antigen may activate T suppressor cells - a process independent of the monocyte). T helper cells and/or soluble

factors then act on B cells to produce antibody with specificity for the original antigen.

T suppressor cells may act by cellular interaction to diminish thymus dependent antibody synthesis and by limiting (in vitro) the generation of cells responsible for cell mediated cytotoxicity. These functions are mediated either by cell-cell contact or by the production of soluble factors which inhibit positive T cell function. T cells appear to influence the class of antibody produced and also its affinity. Thus clones of B cells may switch from the production of IgM antibody to the production of IgG or IgA antibody with the same specificity or, distinct subsets of T lymphocytes may interact exclusively with precursors of cells secreting a particular subclass of antibody e.g. IgG_2 or IgE. Furthermore, in experiments with thymectomized irradiated mice reconstituted with bone marrow cells, it has been observed that the greater the number of thymocytes used in reconstitution the greater the affinity of the antibody produced[105].

A more extensive form of immunoregulation was engendered in Jernes[106] network hypothesis. Instead of a suppressor cell or factor modulating the response, a regulatory system which could operate in the absence as well as the presence of an

antigen was envisioned. In Jernes hypothesis regulation is mediated by anti-idiotype antibodies directed against unique antigenic determinants expressed by antibodies specific for the immunizing antigen. That is, antibodies (or in a broader sense suppressor cells or suppressor factors) are specific for effector cell receptors rather than for antigens themselves - a concept which allows immunoregulation to continue in the absence of an antigen. The potential usefulness of regulating immune responses through interaction with the idiotype of the receptor was accented by Binz and Wigzell[107]. They injected rats with molecules bearing receptors specific for a given set of rat MHC antigens which resulted in the elimination (by development of a form of immunity) of those lymphocytes capable of mounting an alloreaction against cells bearing those MHC antigens for which the receptors used for immunization were specific. These idiotype immunized rats were able to retain skin grafts from rats of the MHC type for which the idiotype bearing molecules were specific.

While it can be expected that antigen exposure will result in a primed state characterized by the appearance of specific T lymphocytes and memory B cells there are situations

in which tolerance develops, i.e. a state in which there is diminution of the immune response with a subsequent challenge with the same antigen. At least four different mechanisms may result in immune tolerance:

1. Clonal abortion - the specific elimination of clones of B and T cells early in their development.

2. Clonal deletion - the removal of clones of mature B and T cells following exposure to tolerogenic forms of antigen.

3. Reversible inactivation of lymphocytes by saturation of receptors with free antigen or antigen antibody complexes.

4. Specific inhibition by the action of primed suppressor cells.

From the foregoing discussion it can be seen that in response to antigen challenge, a number of immunoregulatory events may occur:

1. T cells may promote or suppress the function of B cells or other T cells.

2. B cells may promote or suppress function of T cells.

3. Macrophages may activate T cells and B cells directly.

4. T cells may activate macrophages.

5. T suppressor cells may activate macrophages to produce a nonspecific inhibitor of B cell function.

6. Macrophages may suppress the response of other immunocompetent cells.

It is apparent that the present state of knowledge is the product of several decades of research and while substantial progress has been made in that time, there is now an even greater opportunity to make further advances. New methodologies are rapidly appearing and it is to be anticipated that together with the definition of additional subsets of B and T lymphocytes, the role of the null cell and monocyte will be further evaluated. Evidence in the last few years has suggested the existence of modulatory loops, similar to those recognized in endocrine systems. Mechanistic aspects of this delicate balance which maintains the integrity of the immune system and its responses are the subject of this thesis. In particular a series of studies have been designed to investigate in vitro differences which may exist in immunoregulation between normal individuals and patients with rheumatoid arthritis.

CHAPTER 2

BACKGROUND TO RESEARCH (2)

- IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS.
- ILLUSTRATION OF CLINICAL CONSEQUENCE OF RHEUMATOID ARTHRITIS.
- THE RESEARCH QUESTION.

"to study the phenomena of
disease without books is to sail
an unchartered sea, while to study
books without patients is not to
go to sea at all."

Sir William Osler 1849-1919.

IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS

The immunopathogenesis of rheumatoid arthritis can be conveniently divided into three stages:

1. The initiating event which probably results in the conveyance of an etiologic agent to the joint. Several potential etiologic factors - infection, autoimmunity and genetic predisposition were considered in the last chapter.
2. Immunologic events perpetuating synovitis.
3. The development of pannus from inflamed synovium resulting in joint destruction.

In this chapter the current state of knowledge regarding the immunopathogenesis of rheumatoid arthritis is reviewed and the consequences briefly illustrated. An immunopathogenic mechanism is constructed following an evaluation of observations made on individual components of the immune response.

Immunogenetics:

As alluded to in the previous chapter studies of immunogenetic aspects of rheumatoid arthritis have revealed several interesting facts which may relate to how gene products are involved in the development of rheumatoid arthritis. While no differences generally exist between rheumatoids and normals

in HLA-A, B or C antigens, an increase of HLA DW4[108,109] and a decrease of HLA DRW2 have been observed[108]. Furthermore, a correlation has been noted between DRW3 and high titres of rheumatoid factor, and a questionable association between DRW2 and DRW3 and adverse reactions to remittive drugs (gold and Penicillamine). Finally, patients with HLA B8 and DRW3 (2 genes in linkage dysequilibrium), appear to be particularly liable to the nephrotoxic complications of these drugs.

Enumeration of Lymphocyte Populations and Subpopulations:

Lymphocyte populations have been studied in the peripheral blood, synovial fluid and synovial tissue of patients with rheumatoid arthritis, surface markers proving useful in the identification of subpopulations of these cells. In spite of the imprecision of present methodologies some degree of functional purity has been observed in the defined subsets and several important observations made. Several authors have reported that the proportion (9-18%)[110,111] and absolute number[112] of B lymphocytes in the peripheral blood of patients with rheumatoid arthritis show no deviation from the normal. Problems of correctly identifying cells as B cells or T cells were alluded to in Chapter 1. Even

accounting for these problems it is evident that rheumatoids and normals do not differ quantitatively. Studies of T lymphocytes[110,111] using E-rosetting and anti-T cell antisera have shown similar counts in the blood of rheumatoids and normals. There is no convincing evidence of a consistent fluctuation of T cell levels with disease activity. In respect of the non-B non-T cell population of lymphocytes there have been few reports, although numbers appear to be normal[113]. Thus, B cells, T cells and non-B non-T cells are present in normal numbers in patients with rheumatoid arthritis. Inconsistency regarding the identification of T gamma lymphocytes is likely due to technical factors such as immune complexes, antilymphocyte antibodies and rheumatoid factors, interfering with Fc gamma receptor assays.

The T lymphocyte population in synovial fluid is increased[111], while the B cell proportion is decreased[110,114] when referenced to peripheral blood values. Information regarding the proportion of non-T non-B or null cells is more varied. The explanation for this change in B and T cell proportions is unclear. While the high percentage of T cells may reflect the large number of T cells present in synovium it is also true that T cells are the predominant lymphocyte type in other non-infectious extra vascular fluids[115](lymph, c.s.f. ascites and pleural exudates). The scarcity of B cells

may be due to rapid differentiation into plasma cells, selective destruction or poor migration capability from synovium into synovial fluid.

The lymphocyte has long been recognized as the characteristic cell type of the rheumatoid inflammatory infiltrate, even at times forming lymphoid follicle-like structures. Although cell suspensions have been successfully prepared from synovium, identification of composite cell types has not been as satisfactory. While proportions have varied using immunofluorescent antisera, cyto-adherence techniques and elution and characterization procedures, a trend has emerged indicating that the T cell is the predominant lymphocyte (54-83%)[116,117] while the B cell is present in a proportion of 1-18%[116,118] (using the last mentioned technique). There are objections to each of the three methods. The major objection to the immunofluorescent method is that there is interference by staining of Fc gamma receptors in the surrounding tissue which cannot be controlled for. Fc gamma and C3 receptors on different lymphocyte populations and subpopulations, as well as on other types of leukocytes, reduce the specificity of results obtained using cyto-adherence. Elution methods have the disadvantage that only certain cells are elutable and therefore the subsequent characterization

studies are conducted on only a subset of the true synovial population of lymphocytes. While enumeration studies are valuable they say nothing regarding the functional status of composite cells. These considerations have required the application of different methodologies.

Additional observations have indicated that cutaneous delayed hypersensitivity to various antigens is impaired in some patients with rheumatoid arthritis[119]. Rheumatoid lymphocytes in autologous serum have been shown to perform poorly in the mixed lymphocyte reaction (MLR)[120,121]. Spontaneous cytotoxicity to Chang liver cells is increased, while ADCC and mitogen induced cytotoxicity are normal in peripheral blood of patients with R.A.[122,123,124].

Lymphocyte Function:

Several researchers have observed hyporesponsiveness of rheumatoid lymphocytes to phytomitogens and to specific antigens[125, 126,127]. In particular it has been difficult to stimulate R.A. lymphocytes with either aggregated IgG or immune complexes. The mechanisms by which this hyporesponsive state arises is unknown. However, it has been postulated that lymphocytes are already stimulated by an undefined antigen and so cannot be further stimulated. Of interest in this respect, are the results of experiments conducted on synovial lymphocytes which

show increased ^{3}H-thymidine incorporation. The highest response to phytomitogens occurs in lymphocytes with the highest levels of spontaneous incorporation of ^{3}H-thymidine, suggesting the possibility of two distinct subsets of lymphocytes, one incapable and one capable of further stimulation. The probability that hyporesponsiveness is due to extrinsic factors has been diminished by the observation that prolonged incubation at 37°C (even overnight), or enzymatic removal of adsorbed material using trypsin, neuraminidase or hyaluronidase does not enhance responsiveness[128]. It appears therefore that this functional deficit in vitro is due to an intrinsic abnormality of R.A. lymphocytes or in the way in which they communicate with one another, rather than the result of lymphocyte coating by serum factors. Studies on the number and function of lymphocyte subsets in rheumatoid arthritis have been limited. Work on the Con A suppressor cell and the short lived suppressor cell indicate that neither are active in R.A. Enhancement of PHA induced lymphocyte transformation by Indomethacin has recently been used as evidence for the existence of a prostaglandin producing suppressor cell in normal as well as rheumatoid subjects[129]. Although this observation is of interest in

view of the important role prostaglandins play in the regulation of the immune response[130], considerable work is required to evaluate its true significance.

Lymphokines:

Lymphokines as previously described are biologically-active lymphocyte-derived soluble factors which play a vital role in the immune response. If R.A. is viewed as a disorder in which tissue lesions result from the direct participation of persistently activated immune cells and their products, then the place of lymphokines in this scheme requires definition. Although thoracic duct drainage[131] necessitates the removal of fluid as well as lymphocytes, the beneficial effects of this form of therapy have been used as evidence that lymphocytes are directly involved in the rheumatoid process. Furthermore, lymphokines have been found in rheumatoid synovial fluid and cell supernatants[132], and when injected into rabbit joints have produced chronic synovitis[133]. Lymphokines in various situations have been shown to produce cell migration inhibition, mitogenesis, monocyte and osteoclast activation, cell cytotoxicity and vascular changes. While the extent to which lymphokines contribute to the immunopathology of rheumatoid arthritis is speculative, there is reasonable evidence to support their presence in the rheumatoid inflammatory process and the importance of their biological activity.

Rheumatoid Factors:

Rheumatoid factor (R.F.) is an antibody directed against the Fc region of IgG. While this is usually an IgM antibody, other classes have been recognized. Even IgM RF is not a homogenous entity, several fractions with separate antibody specificities being recognized[134]. The mechanism by which self tolerance to IgG is lost in R.A. is not known, although there are several postulates. There may be direct stimulation of non tolerant B cells or loss of suppressor cell activity. Polyclonal B cell activation may result in the activation of auto antibody classes or alternatively immunogenic determinants on complexed, fragmented or altered monomaric IgG may be exposed. One area that has been of significant interest is the possibility that rheumatoid factor is the product of an immune response against an antigenic determinant which crossreacts with IgG[135,136]. Although no antigen has been defined it has been observed that purified IgM rheumatoid factor will react with the nucleosomes of normal cells, a phenomenon requiring the presence of DNA and core histone. This cross reactivity has not been noted by several researchers and may relate to the inhibitory effect that excessive quantities of IgG have on this "anti-nuclear" activity of R.F. Therefore it is possible

that the aetiologic event results in the generation of an intracellular antigen to which an antibody (IgM) response is mounted. Then because of crossreactivity the antibody reacts with the Fc region of IgG. Since the antigen is not irradicated and IgG is in constant supply, the immune response is perpetuated.

Immune Complexes:

Studies of immune complexes present in rheumatoid synovial fluids have demonstrated that a large proportion contain anti-IgG antibodies, particularly of IgG class[137,138]. However, rheumatoid factors have been demonstrated in the synovial fluids of patients with several non-rheumatoid chronic inflammatory disease states. It is possible that differences in clinical manifestation are due to some characteristic of the rheumatoid factor such as site of production, immunoglobulin subclass, the antigenic determinants concerned, antibody specificities and various other biological properties. Evidence suggests that most individuals have the capacity to produce anti IgG antibodies following immunization by IgG-containing immune complexes, or by naturally occurring polyclonal B cell activators. The structural similarity of anti IgG from unrelated individuals has suggested a genetic influence on

anti-IgG production[139]. Anti IgG antibodies have two important properties. Firstly, they react with an antigen present in serum (IgG) and secondly, the auto antigens reside on the antibody molecule itself. The observation that most IgG in rheumatoid synovium is produced in situ[140] can be related to the finding of IgG anti-IgG complexes, the formation of which are likely facilitated by the presence of high local concentrations of auto antibody and low concentrations of normal IgG. Furthermore, some IgG anti-IgGs preferentially self associate[141] forming IgG anti-IgG - IgG anti IgG complexes. The generation of immune complexes is of more than just casual concern since it is the anti IgG containing complexes which can activate complement and initiate the sequence which ultimately leads to joint damage. Further evidence for the pathogenetic importance of anti IgG comes from the observation that IgG-anti IgG complexes can cause mesenteric arteritis in rats[142] and also are found in arteric lesions in patients with rheumatoid arthritis. Furthermore, patients with severe disease tend to have higher titres of rheumatoid factors in their blood and show evidence of complement consumption[143]. One final point to be made regarding rheumatoid factor is that while it has been incriminated in the pathogenesis of rheumatoid arthritis, protective effects for the presence

of rheumatoid factor have been shown in other settings. R.A. anti-IgG can attentuate serum sickness[144], reduce complement mediated cell lysis[145] or cell mediated cytotoxicity[146], or inhibit heterologous anti-human IgG antibody production[147]. How these in vitro observations relate to the immunologic events which produce this clinical entity is speculative.

Auto antibody production in rheumatoid arthritis is not confined to rheumatoid factor. Several auto-antibodies of different classes to collagen of varying types have been detected in R.A. synovial fluid and to a lesser extent in serum[148,149]. The finding of these antibodies is not peculiar to R.A., being found in S.L.E., P.S.S., psoriatic arthritis and several other arthropathies. It is unlikely that these auto antibodies initiate the synovitis but more likely that they arise as a result of stimulation of immunocompetent cells by antigens derived from breakdown products of autologous denatured collagen. In this respect they play a role in perpetuating the synovitis which was initiated by a different antigen. Antinuclear antibodies are found in a high percentage of patients with rheumatoid arthritis.

Immune complexes have been studied in synovial membrane and fluid, collagenous tissues as well as in blood. Although complexes are detectable in the circulation they are more

readily appreciable in extravascular areas, and in particular within joints. Identification of complexes has been achieved by a number of different methods (C1q binding, precipitation with polyclonal IgM-RF, radial diffusion etc.). However, the specificities of IgG rheumatoid factors have still not been fully investigated, and the mechanism whereby synthesis of rheumatoid factor is perpetuated not evaluated. Evidence suggests that a local immune response occurs in rheumatoid synovial membrane, either because of the presence of self replicating foreign antigens (e.g. virus) or of auto antigens responsible for the maintenance of a local autonomous antibody response, resulting in the formation of various types of immune complexes, mainly IgG anti-IgG but also including IgM complexes and self associating anti-IgG.

From elegant studies conducted in rabbits, Jasin has observed the deposition of immune complexes in collagenous tissues. Early experiments[150] indicated that local antibody production continued for two months after the intra articular injection of soluble antigen and moreover, the majority of the antigen was sequested, not within the synovial membrane but rather in avascular collagenous structures such as articular cartilage, menisci and tendons, where it was irreversibly trapped. Furthermore, the antigen

was complexed with complement and IgG, complexes being most frequent in the superficial area of tissues close to the articular surface. These findings have subsequently been confirmed by an independent group of workers[151]. A search for immune complexes in rheumatoid collagen has revealed their presence in 92% of patients with classical disease[152]. However, complexes have also been found in other inflammatory and non-inflammatory joints, although less frequently. Using different techniques, the presence of complexes in rheumatoid collagenous tissues has been confirmed[153,154]. These trapped immune complexes have been shown to be capable of generating mediators of acute inflammation when incubated with complement[155,156]. The presence of an inaccessible antigen trapped in an immune complex has major implications in the possible pathogenesis of rheumatoid arthritis. Frustrated phagocytosis will result in the continuous release of mediators of inflammation in a nonspecific manner, while the continued presence of antigen may lead to the accummulation of sensitized lymphocytes. Finally, the presence of acute inflammatory cells within damaged collagenous joint tissues may result in the release of factors which are chemotactic for other cells and possibly for pannus.

There is also evidence of a pathogenetic role for immune complexes in the development of rheumatoid vasculitis where they have been found in dermal vessels[157] and the vasa nervorum of vasculitic lesions[158].

Immune complexes have been demonstrated in the circulation of some patients with rheumatoid arthritis[159,160] and it is deposition of these complexes[158] which is thought to account for some of the extra-articular features of the disease e.g. nodules, nail fold haemorrhages, peripheral neuropathy, skin necrosis, episcleritis and lung lesions. There are several methods of immune complex detection, each recognizing complexes with different physical and immunochemical composition. While different complexes may be detected by each method it is possible that different methods at least in part identify different facets of the same complexes, while also failing to detect other complexes similar in other respects. The relationship between circulating immune complexes and disease severity, activity and prognosis continues to be evaluated.

Complement Activation:

The classical and alternate pathways of activation of complement and the control proteins C1 INH, C3bINA and BIH have been studied in rheumatoid synovial fluid and peripheral blood. Apart from occasional episodes of hypocomplementemia

during disease exacerbation, the total hemolytic complement (CH_{50}) of rheumatoid peripheral blood is usually normal or elevated[161,162]. This is in contrast to the decreased complement activity (compared with osteoarthritic joints) noted in synovial fluid of seropositive rheumatoids[163]. However, the definition of a normal range for synovial fluid complement has been unsatisfactory. Studies have indicated a hypercatabolism of C_3, C_4 and factor B indicating classical but also alternate pathway activation[164,165]. Immune complex induced activation of the classical pathway may represent the main and early pathogenetic event with secondary activation of the alternate pathway. The control proteins C3bINA and BIH have been reported as being depressed in the synovial fluid of seropositive rheumatoids, while only C3bINA is depressed in seronegative. In contrast, in rheumatoid serum (in association with normal CH_{50} levels) supranormal levels of C3bINA and BIH suggest that excessive systemic complement activation is prevented by circulating control proteins[166].

Phagocytic Cells.

Immunohistochemical studies have revealed the presence of immunoglobulin and complement inside phagocytic cells within synovial membrane and in the synovial fluid[140]. Since monocytes[167] and polymorphonuclear leucocytes (PMN) are common in rheumatoid synovial fluid, their role in enzyme mediated joint damage requires consideration[168]. Studies on PMN's in R.A. have usually demonstrated decreased adherence[169], chemotaxis[170] and phagocytosis[171] in vitro. Not all studies have been conducted on drug free patients and therefore it has been questioned whether in some cases defective PMN function is a consequence of treatment rather than disease. Two possibilities have been raised to account for this functional deficiency, (a) the prior ingestion of immune complexes and (b) C3b receptor blockade on the surface of the PMN. At least in synovial fluid the latter hypothesis is favoured. While certain functional defects have been shown, PMNs are nevertheless capable of releasing enzymes such as liposomal proteases, elastase, collegenase, cathepsin G, cathepsin D and superoxide radicals, all of which are able to inflict tissue injury[172]. In spite of in vitro studies demonstrating hypofunction, the observation of phagocytosed material in PMNs shows that phagocytosis can be achieved in vivo. Research into the functional capabilities of rheumatoid monocytes promises to provide valuable information about this cell which is not only an

immunocompetent regulator but also an effector cell capable of phagocytosis, and the release of enzymes some of which can degrade articular cartilage.

Fibrin.

Pertinent to the present discussion are observations regarding elements within the joint capsule which may result in the observable pathologic endpoints of proliferation and destruction. Fibrin is extensively deposited in rheumatoid joints being found free in synovial fluid, on synovial pannus and cartilage, as well as within phagosomes. While fibrin deposition occurs in other inflammatory arthritides it is persistent in rheumatoid arthritis. Persistence appears to be due to continued overproduction of fibrin, rather than inhibition of fibrinolysis. The stimulation for this overproduction may be activation of the coagulation process by immune complexes or lymphocytes. A pathogenetic role for fibrin has been suggested, similar to that postulated for the Masugi nephritis model[173]. By analogy in rheumatoid arthritis fibrin may result directly or indirectly in pannus formation, fibrous thickening of the synovium, swelling and proliferation of synovial lining cells and vascular endothelium, induration of the synovium, emigration and degeneration of PMNs, PMN mediated cartilage destruction and lymphoedema causing synovial

swelling and joint effusion[173-176]. It is evident that further research is required to define the problem as it exists in rheumatoid arthritis.

Synovium.

Rheumatoid synovium has several characteristics. It is a multilayer structure easily distinguishable from normal synovium, in which there is a proliferation of small blood vessels and collagen, together with a heavy infiltrate of plasma cells and lymphocytes. The invasive nature of pannus has been demonstrated in rheumatoid synovial explants[177]. Furthermore, inflamed synovium can itself produce collagenase[178]. The intracellular regulator of collagenase production is unknown although $_2$-macroglobulin is one of its extracellular inhibitors.

The destruction of cartilage appears to be the consequence of primary degradation of proteoglycan by proteinases, followed by breakdown of exposed collagen by collagenases[179]. Proteinases arise from three sources: synovial fluid cells, the synovium and chondrocytes. Proteinase released from PMNs has been the most extensively studied. Elastase and cathepsin G are contained in the azurophilic granules of the PMN. Whether release is due to activation on attempted phagocytosis (regurgitation during feeding and frustrated phagocytosis) or due to the death of

many PMNs entering the joint and dying, is not known. These serine proteinases readily hydrolyse proteoglycan and may degrade insoluble collagen[180]. Knowledge regarding synovial and chondrocyte proteinases is much more limited. Although both have been shown capable of producing proteinases, their quality and functional activity are unknown.

Prostaglandins and Superoxide Radicals.

Two relatively new areas of intense interest are those of prostaglandin[181] and superoxide radical release[182]. Prostaglandins have been shown to have profound in vitro effects on the inflammatory response, but several studies have been critisized on the basis of using supraphysiological doses of these compounds. Furthermore, different classes of prostaglandins and/or different concentrations appear to produce differing responses. Of interest when considering R.A. synovitis are the observations that PGE_1 injected intra-articularly into rabbit joints causes cartilage destruction[183], while prostaglandin synthetase inhibitors are capable of reducing bone resorption by R.A. synovium in the mouse calvarium assay system[184]. While this suggests a role for prostaglandins in bone resorption, it is nevertheless indirect evidence. The presently held view by many rheumatologists that nonsteroidal

anti-inflammatory drugs often fail to prevent progression of bone and cartilage changes suggests that direct comparison cannot be drawn between the animal model and humans. Since prostaglandins are ubiquitous and their effects on the inflammatory response so varied, significant advances can be anticipated in this area of research. The destructive capability of free oxygen radicals released from phagocytic cells provides insight into another mode of tissue injury. Phagocytes are capable of generating a number of active molecules by the partial reduction of oxygen. Oxygen is initially reduced to superoxide (O_2^-) by a membrane associated flavoprotein that is dormant in the resting cell but is activated when a cell encounters an appropriate stimulus. The reducing agent for this reaction is NADPH. A large part of the O_2^- produced is converted to H_2O_2 in a subsequent dismutation reaction. Much of the H_2O_2 is delivered into phagosomes or into the surrounding medium, a portion diffusing into the cytoplasm where it is detoxified by the glutathione-peroxidase-glutathione-reductase system. The O_2^- forming reaction and glutathione reductase both generate $NADP^+$ in the course of their activity; thus $NADP^+$ is converted back to NADPH by the hexose monophosphate shunt. O_2^-, H_2O_2 and other oxygen derivatives are considered part

of the antimicrobial system of phagocytes but also thought capable of inflicting injury on host tissues. Thus superoxide radicals are believed to play at least some part in the pathogenesis of rheumatoid arthritis.

Postulated mechanism of tissue injury.

Having reviewed present knowledge regarding components of the immune response in R.A. it is convenient to attempt to integrate it into a construct which may approximate events occurring within joints of patients with the disease. It can be conceptualized that an antigen (possibly a virus) gains access to the joint in a susceptible host by failure of the normal mechanisms of immune surveillance. Once in the joint the antigen becomes distributed in synovium and cartilage and if a virus, self-replicates. An immune response is mounted by monocytes processing the antigen and presenting it to T cells, which in turn activate B cells. Transformation into plasma cells occurs and antibody is produced. Lymphokine release from activated lymphocytes augments the inflammatory response. Because of cross-reactivity, the antibodies produced have rheumatoid factor activity. Immune complexes of several different types are formed and complement is activated, resulting in vascular changes and the accummulation of polymorphonuclear leukocytes. These, together with activated macrophages attempt to phagocytose the complexes. Some are

engulfed. In any event collagenases, elastases, cathepsins D and G, prostaglandins, superoxide radicals and several other biologically active agents are released by a combination of reverse endocytosis, frustrated phagocytosis and cell death. These substances produce degradation of proteoglycan and collagen which in turn results in the production of antibodies directed against collagen fragments. Fibrin and other active compounds enhance vascularity, synovial hyperplasia, pannus formation and the production of joint effusions. Perhaps because the foreign antigen complexes are sequested or because antigen self replicates, or because antibodies arise against self antigen (immunoglobulins) or because there is a failure of immunoregulation, inflammation is perpetuated. As a result pannus is formed which invades the peripheral articular cartilage causing progressive joint destruction.

From the foregoing discussion it is evident, that while a plausible sequence of events to account for several observations can be constructed, there remain many areas in which knowledge of the exact process is incomplete. It is particularly difficult to dissect events of primary immunopathogenetic importance from those which occur as a consequence of an established immune response. Furthermore, it is not clear which, if any, of the events is of greatest importance, i.e. B cell hyperactivity, T cell infiltration, fibrin deposition, complement consumption, immune complex formation,

activity of phagocytic cells etc. Nevertheless, the construct described is the most likely sequence of events in the light of current knowledge.

ILLUSTRATION OF CLINICAL CONSEQUENCES

OF

RHEUMATOID ARTHRITIS

Since the next chapter describes the development of experimental methods and their standardisation in patients with rheumatoid arthritis and normal subjects, it is necessary to define those features by which the disease is identified. In the classical case the condition is unmistakable. There exists in a patient, normally female, a widespread, predominantly peripheral, symmetrical polyarthritis with prolonged and distressing early morning stiffness, subcutaneous nodules, erosive joint changes on x-ray, elevated erythrocyte sedimentation rate and other laboratory evidence of chronic inflammation; positive tests for "rheumatoid factor" in the blood and characteristic (but not unique) histological appearances in the synovium and subcutaneous nodules. However, many cases exist with incomplete or atypical features, as well as other non-rheumatoid arthropathies mimicking the disease. Although diagnostic criteria are not ideal the American Rheumatism Association[185] has drawn up inclusion (Table I) and exclusion (Table II) criteria for the diagnosis of the disease. No one of the eleven criteria can completely define the disease, it being necessary to recognise the presence of several criteria in combination to identify the disease. The probability of an individual having rheumatoid arthritis varies with the number of criteria present (Table 3). The

TABLE 1.

American Rheumatism Association

Diagnostic Criteria for Rheumatoid Arthritis.

1. Morning Stiffness.

2. Pain on movement of tenderness in at least one joint (observed by a physician).

3. Swelling due to soft tissue or fluid in at least one joint (observed by a physician).

4. Swelling of at least one other joint within three months.

5. Symmetrical joint swelling with the same joints affected on both sides of the body at the same time.

6. Subcutaneous nodules over bony prominences on extensor surfaces.

7. X-ray changes typical of rheumatoid arthritis.

8. Positive agglutination test (i.e. demonstration of rheumatoid factor by any method which in two laboratories has been positive in not more than 5 per cent of normal controls.

9. A poor mucin precipitate from synovial fluid with shreds and a cloudy solution.

10. Characteristic histological changes in synovial membrane: villous hypertrophy; proliferation of synovial cells; chronic inflammatory infiltrate with a tendency to form lymphoid nodules; deposition of compact fibrin and foci of cell necrosis.

11. Characteristic histological changes in nodules; i.e. granulomatous foci with central zones of necrosis and peripheral fibrosis and chronic inflammatory cell infiltrate.

TABLE II.

Exclusions from classification of rheumatoid arthritis.

1. A typical rash of systemic lupus erythematosus.
2. High concentrations of L.E. cells.
3. Histological evidence of periarteritis nodosa.
4. Dermatomyositis or muscle weakness.
5. Definite scleroderma.
6. A characteristic clinical picture of acute rheumatic fever.
7. A characteristic clinical picture of gouty arthritis.
8. Tophi.
9. A characteristic clinical picture of acute infective arthritis.
10. Histological or bacteriological evidence of joint tuberculosis.
11. A characteristic clinical picture of Reiter's syndrome.
12. A characteristic clinical picture of the shoulder hand syndrome.
13. A characteristic clinical picture of hypertrophic pulmonary osteoarthropathy.
14. A clinical picture characteristic of neuro-arthropathy.
15. Homogentisic acid in the urine.
16. Histological evidence of sarcoid or a positive Kveim test.
17. Multiple myeloma.
18. Characteristic skin lesions of erythema nodosum.
19. Leukaemia or lymphoma.
20. Agammaglobulinaemia.

TABLE III. CHARACTERISATION BY DIAGNOSTIC CRITERIA.

Categories	Number of Criteria Required	Minimum Duration of Continuous Symptoms
Classic Rheumatoid Arthritis	7 of 11	6 weeks (1-5)
Definite Rheumatoid Arthritis	5 of 11	6 weeks (1-5)
Probable Rheumatoid Arthritis	3 of 11	6 weeks (one of numbers 1-5)

diagnosis of classical rheumatoid arthritis requires the presence of 7 or more criteria and represents the highest probability of the presence of the disease. Definite (5), Probable (3) and Possible (2) represent progressively lower probabilities. In the studies to be described only patients with classic or definite rheumatoid arthritis have been selected. This selection represents a compromise between a need to be certain of the diagnosis and reluctance to study only a subset of the disease. Nevertheless the ARA criteria are the current "gold standard" of diagnosis and are used as entry criteria for the majority of studies reported in the literature.

Sir William Osler's (Figure 9) often quoted statement (noted at the beginning of this chapter), illustrates the importance of diseased individuals in focusing educational and research activities. The ubiquity and relative frequency of rheumatoid arthritis previously eluded to, in themselves justify a necessity independent from intellectual curiosity for studying the disease rheumatoid arthritis. While the disease completely cripples or completely remits in < 10% of affected individuals, in the majority it follows a persistent relapsing and remitting course showing a tendency to progression. The clinical features of the disease have been thoroughly studied, are described in standard texts and are

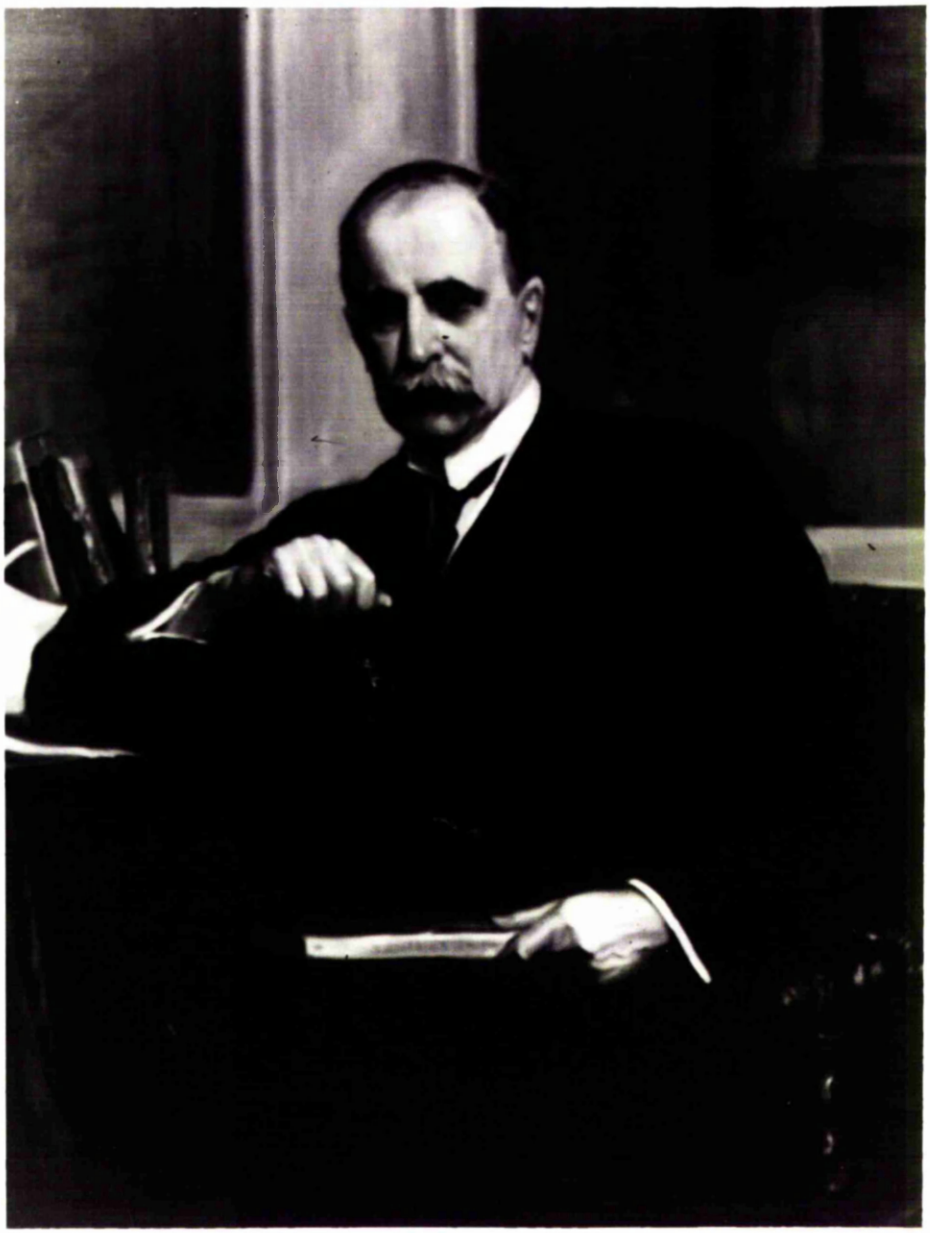


FIGURE 9 - SIR WILLIAM OSLER (1849-1919)

not the principle focus of this thesis. Although infrequent the deforming arthropathy (Figure 10), necrotizing scleritis (Figure 11) and digital gangrene (Figure 12) serve to illustrate some of the most severe clinical consequences and the systemic nature of this common disorder.

The cost of providing health care for patients with arthritis and the cost in terms of lost productivity as a consequence of restricted activity has been considerable[186, 187]. These costs continued to increase on an annual basis and thus provide a significant stimulus to define ways in which the disease and its consequences may be reduced, and sufferers of the disease returned to full or part-time employment through research activities directed at comprehending the nature of the disease and development of new and more effective methods of treatment.

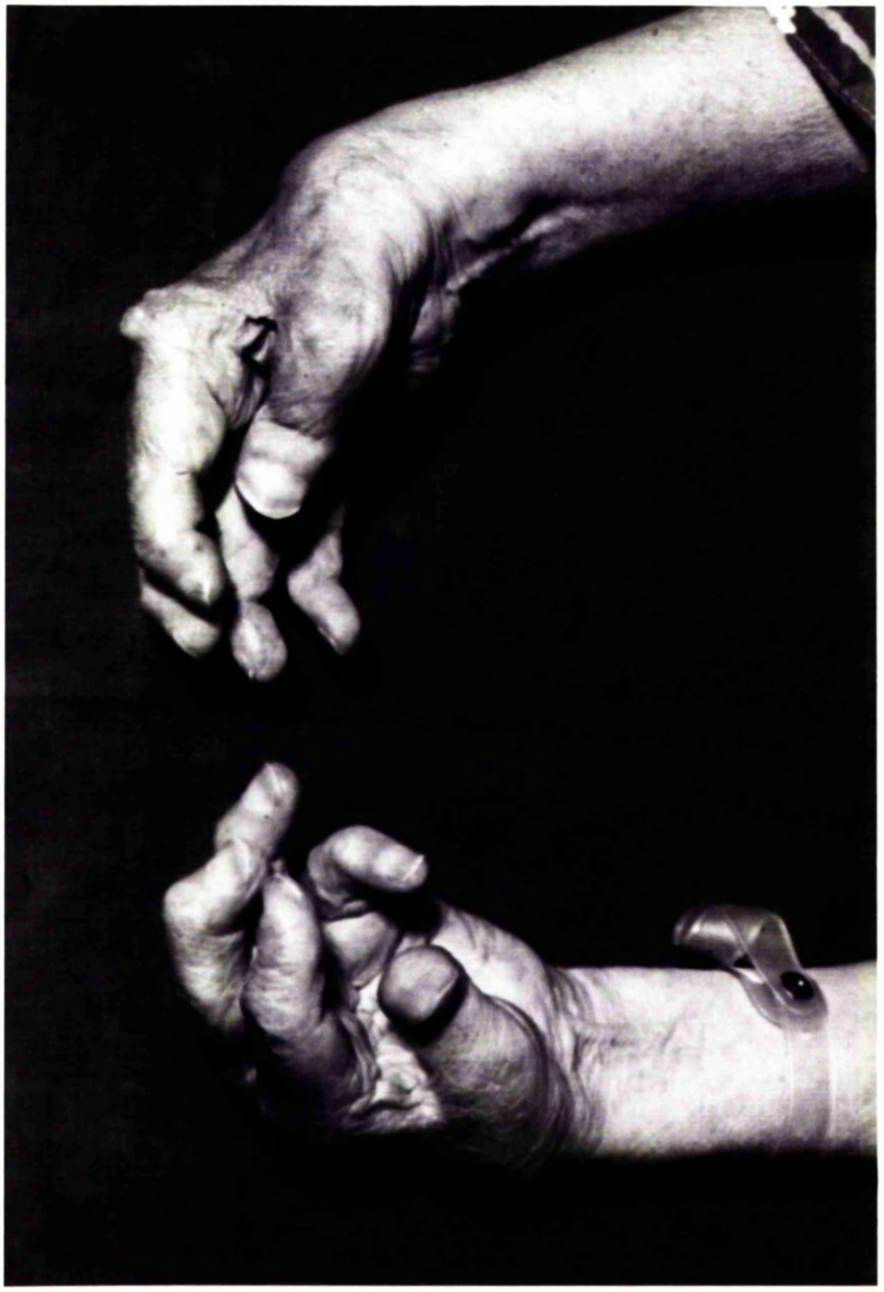


FIGURE 10 - SEVERE DEFORMING SEROPOSITIVE RHEUMATOID ARTHRITIS IN A 75 YEAR OLD FEMALE.

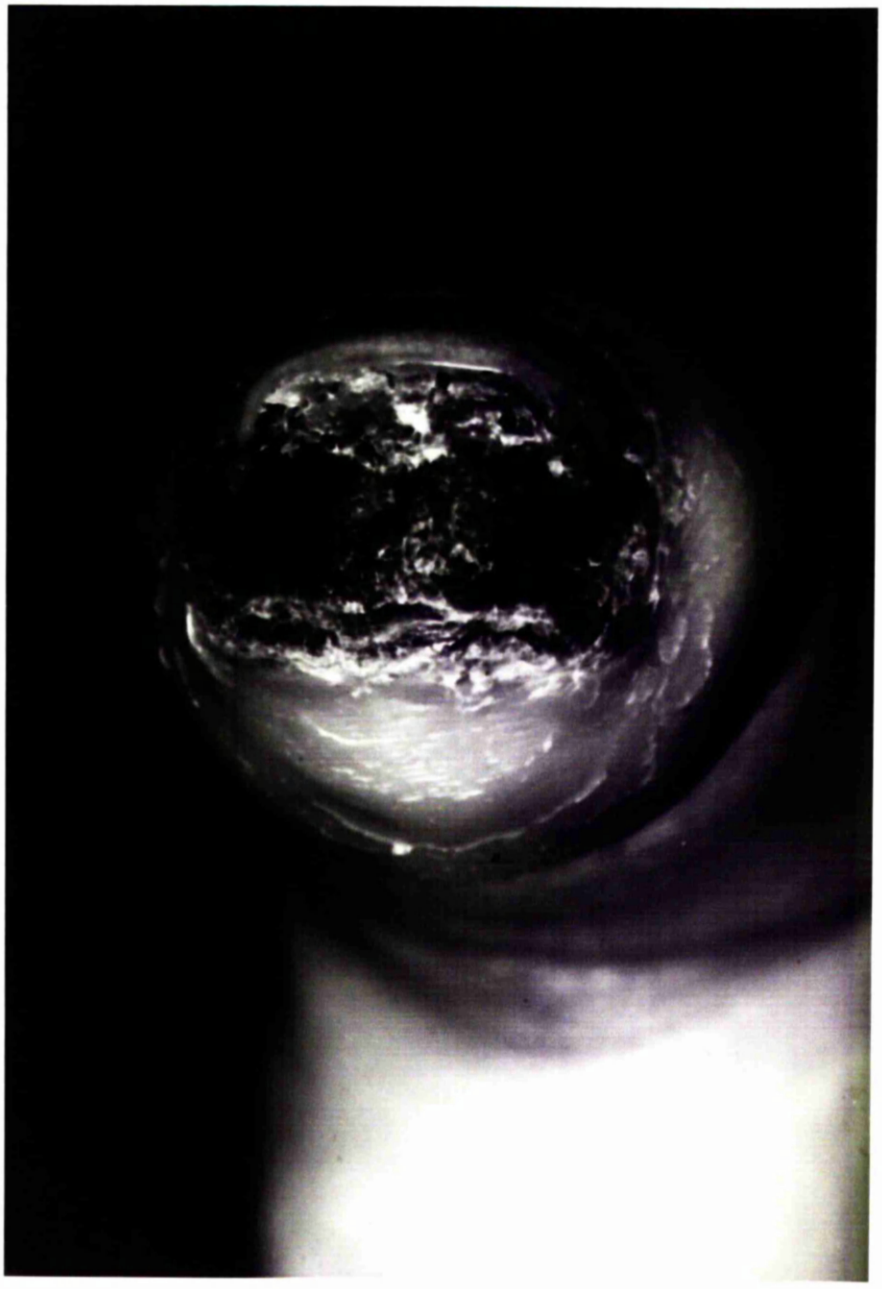


FIGURE 11 - RHEUMATOID VASCULITIS PRODUCING DIGITAL GANGRENE IN A MIDDLE AGED MALE.

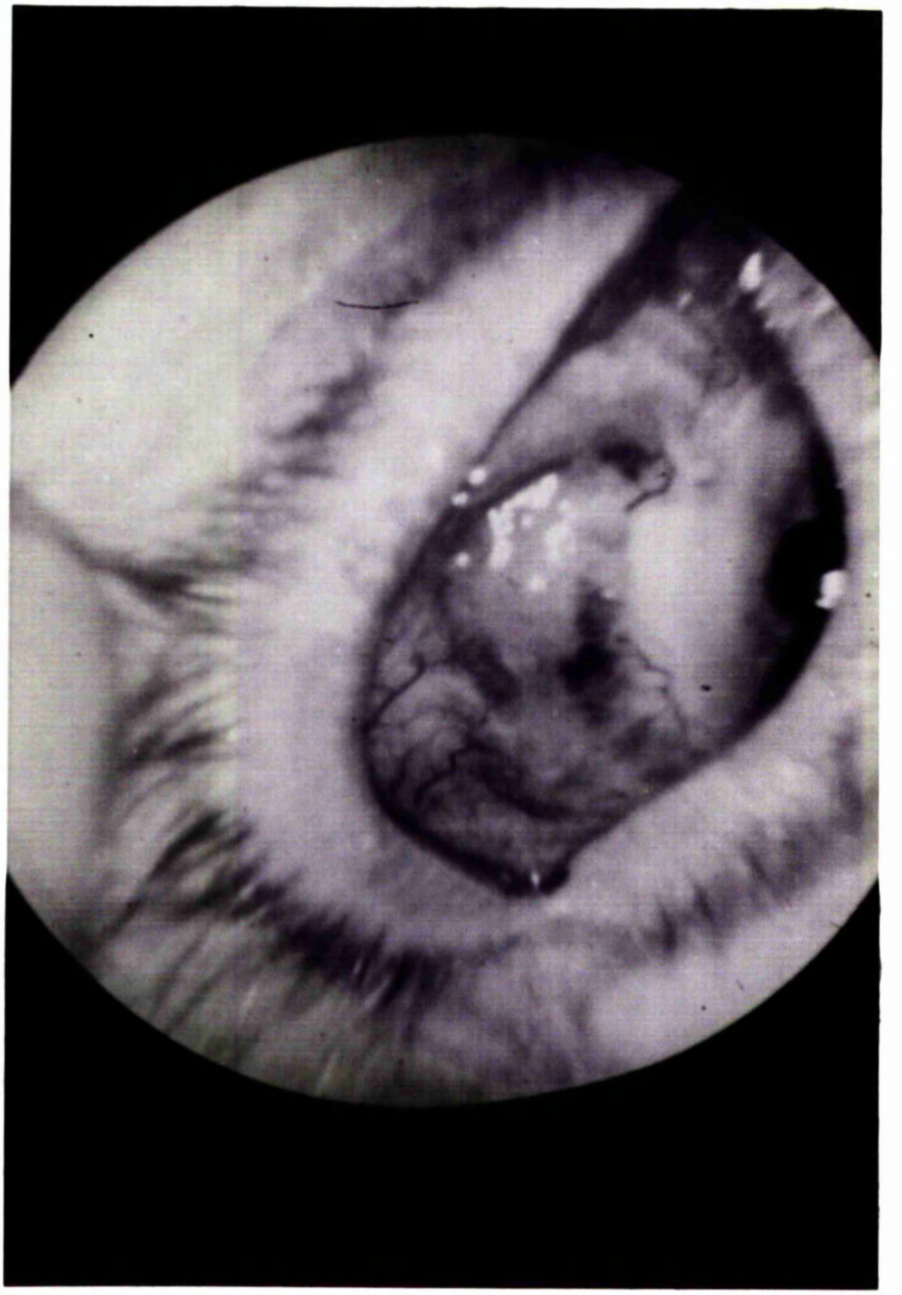


FIGURE 12 - OCULAR RHEUMATOID - ILLUSTRATING NECROTISING SCLERITIS.

THE RESEARCH QUESTION

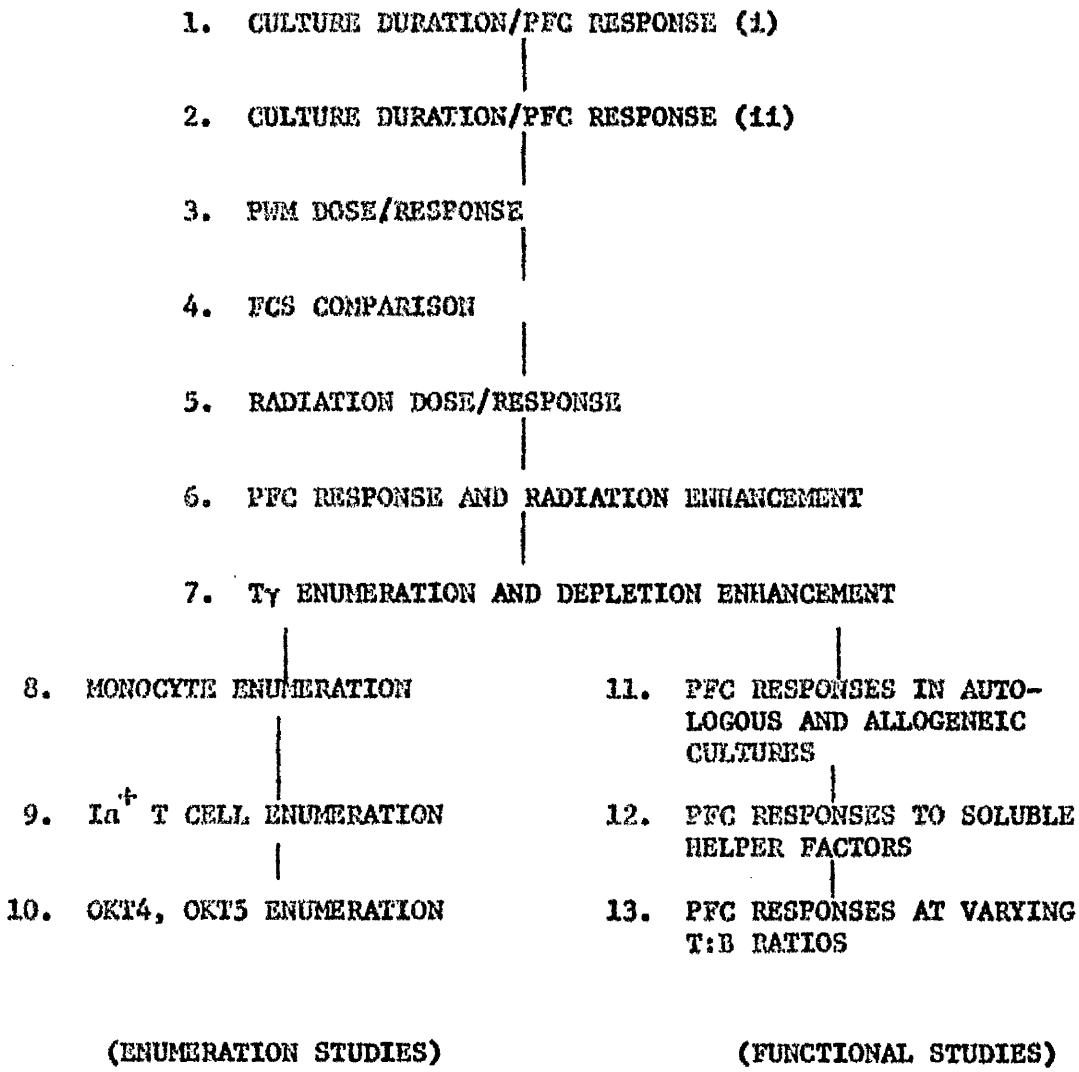
Following this literature review it was apparent that while immunoresponsiveness in rheumatoid arthritis had received considerable attention, there was a paucity of information regarding regulation of the observed responses. Furthermore several available methodologies had not been applied in patients with this disease and promised to provide valuable information possibly of aetiologic and/or pathogenetic importance. Therefore the following research question was formulated:

"Since the manifestations of rheumatoid arthritis result from chronic activation of the immune response, can a defect in immunoregulation be identified in vitro with available methodologies, and if present what is the nature of such a defect."

In view of the systemic characteristics of rheumatoid arthritis and the circulation of immunocompetent cells between intra and extra-vascular areas, the peripheral blood provided a convenient site of sampling to obtain mononuclear cell populations for in vitro studies. A series of controlled studies was planned to examine both quantitative and qualitative aspects of immunoregulation (Figure 13). Studies 1-5 described in Chapter 3 are pilot studies directed at standardising the adopted system. The effects of radiation (Study 6) on immunoglobulin synthesis by B lymphocytes

and physical removal (Study 7) of subpopulations of T lymphocytes are examined in Chapters 4 and 5 respectively. The enumeration of Tγ cells (Study 7), monocytes (Study 8), Ia^+ T cells (Study 9) and $OKT4^+/OKT5^+$ T cells (Study 10) is described in Chapters 5, 6, 7, and 8. Chapter 9 describes the results of experiments using both autologous and allogeneic culture systems (Study 11), while in Chapter 10 the effects of soluble helper factor in rheumatoid and normal cultures are compared (Study 12). In the final study (Study 13), described in Chapter 11, comparison is made between cultures containing different ratios of B and T lymphocytes.

FIGURE 13 - STUDY DESIGN.



CHAPTER 3

DEVELOPMENT OF A STANDARDISED SYSTEM

- BACKGROUND TO STUDY METHODS

- STANDARDISATION EXPERIMENTS

"measure what is measurable
and what is not measurable
make measurable".

Lord Kelvin 1824-1907

BACKGROUND TO STUDY METHODS

- Separation of mononuclear cells.
- Separation of subsets of lymphocytes.
- Measurement of lymphocyte activation.
- Polyclonal B cell activators (PBA).
- Manipulation of subpopulations.
- Plaque assays.

In the first part of this chapter the literature pertaining to methods used in studies 1-5 will be reviewed, since it was necessary to select and modify techniques from several different laboratories in order to produce the final study design. In the second part studies 1-5 are described, and the optimal conditions for subsequent experiments defined. The method finally selected involved separating mononuclear cells (lymphocytes and monocytes) from fresh heparanized blood, obtaining preparations of B and T lymphocytes from the resulting cell fraction, culturing cells and introducing them into an indicator system that measured immunoglobulin synthesis. By manipulating subpopulations of lymphocytes the effect of T helper and T suppressor cells on the B cell response could be observed.

Separation of mononuclear cells:

Following recognition that the lymphocyte was a potentially immunocompetent cell, attempts at study were hindered by lack of an adequate method to obtain pure lymphocytes from whole blood. Albumin density gradients, gelatin sedimentation, magnetic techniques relying on the phagocytosis of iron particles by granulocytes and monocytes, and adhesion of granulocytes and monocytes to glass wool or glass beads were some of the earlier

methods used. Fichtelius[188] was probably the first to use cotton wool filtration - a technique which Lamvik[189] refined. The procedure had three stages. (1) Sedimentation of erythrocytes with Dextran. (2) Removal of platelets from plasma by low speed centrifugation and (3) Separation of granulocytes on cotton wool columns. With this method separation was complete although 10% of the cells were damaged and the yield was variable. This method was utilized in several laboratories prior to the development of more sophisticated centrifugation techniques.

When fresh heparanized blood is centrifuged in at least a 1g gravity field, the cellular content sediments out as dictated by the physical properties (density) of differing cell components. Red cells and granulocytes, then lymphocytes and platelets are packed into distinct layers at the bottom of the tube, the plasma lying above. If a substance, with a density intermediate between any two layers is present under the whole blood layer prior to centrifugation, particles with a greater density will pass through while those with a lesser density will not. It can be seen that the density of this intermediate substance is critical for effective separation. In a detailed study, Boyum[190] evaluated a separation technique with Isopaque-Ficoll (density 1.077) as the

intermediate. He made several observations. (1) The yield of mononuclear cells was almost one hundred per cent when blood was diluted with saline prior to centrifugation over Ip-Ficoll. (Aggregates of granulocytesin undiluted blood pulled down some lymphocytes and decreased the yield). (2) Heparanization at 10 I.U/ml of blood was adequate. (3) Some mixing and diffusion at the interface between plasma and Ip-Ficoll occurred and mononuclear cells often sedimented a few millimetres down into the Ip-Ficoll mixture. (4) Platelets sedimented with mononuclear cells and did not penetrate the Ip-Ficoll. (5) Centrifuge temperature possibly by affecting Ip-Ficoll viscocity altered the efficiency of separation and the yield of lymphocytes. (6) Ip-Ficoll density greater than 1.077 ensured a high yield of mononuclear cells, while low density paradoxically increased the RBC contamination possibly by reducing Ficoll induced cell clumping at the interface. (7) In a comparison of anticoagulants Heparin gave a higher lymphocyte yield than EDTA, although RBC contamination was higher. (8) When Ip-Ficoll osmolarity was increased lymphocytes tended to sediment with RBC's and granulocytes, leaving a monocyte enriched layer above the Ip-Ficoll - an observation which could not be used successfully to obtain a

a pure monocyte preparation. (9) Viability of the resulting cell populations was evaluated using several methods (Trypan blue staining, incubation with L.E. serum, phagocytosis of L.E. bodies by granulocytes and phagocytosis of black and yellow ink particles). Mononuclear cell viability was greater than 99% indicating that this method of separation did not have a detrimental effect on cell viability. (10) When the system was standardized for centrifugal force, temperature, dilution of blood and for properties of Ip-Ficoll the results were not appreciably affected by change in the volume of cell suspension or tube diameter. (11) With this method the mononuclear cell fraction contained less than 0.2% granulocytes and less than 10% erythrocytes. Four years later Jondal et al[191] reported their experience with Ficoll-Isopaque gradients but chose $4^{o}C$ as the centrifuge temperature of choice and did not dilute blood prior to overlaying. In 12 normal subjects they recovered relatively pure (less than 5% contamination with granulocytes and monocytes) preparations of lymphocytes, 52-81% of which were identified by rosetting as T cells. Their cell yield per ml of blood was not defined by the resultant population of cells were described as healthy and little clumping was observed.

From these experiments it was evident that the necessary methodology existed to obtain relatively pure preparations of viable lymphocytes from whole blood. The technique of Jondal et al[191] was adopted after preliminary studies in London indicated a high yield of viable (greater than 99%) lymphocytes with minimal contamination.

Separation of subsets of lymphocytes.

In order to study interactions occurring between subsets of immunocompetent cells it was necessary to obtain relatively pure preparations of B and T lymphocytes whose viability and surface characteristics had not been knowingly altered. Eisen[192] described a nylon wool method of isolating pure (greater than 99%) T cells from human peripheral blood, while Greaves[193] obtained purified preparations of B and T lymphocytes from tonsil cell suspensions. Trizio[194] evaluated the efficacy of this system using mouse spleen cells, introduced the concept of detaching adherent cells from the column by mechanical agitation and determined the functional capability of derived populations in a murine model. All these separation methods rely on the physical property of B lymphocytes (and monocytes) to adhere to nylon fibres in the column, while T lymphocytes form no such adherance and are easily washed off. B cells (and

monocytes) on the other hand require to be mechanically eluted by a combination of agitation of the nylon wool and fluid flow, a process which does not traumatize the cells or alter their viability. Werner et al[195] described a method using 5 ml syringes packed with 0.5 grams of nylon wool, which could separate up to $100x10^6$ lymphocytes. Their technique involved three incubations. (1) Prior to loading lymphocytes on to the primed column. (2) Post loading. (3) Post elution of T cells. They used phosphate buffered saline and/or AB serum to prime and run the column. With this method the yield was approximately 50% of the filtered load. (B cell yield 30% of original B load, T cell yield 60% of original T load.) 50-80% of the adherent fraction had surface immunoglobulin as identified by anti-Ig (immunofluorescence), while the average B cell contamination of the nonadherent T cell preparation was 3.6%. Cell viability was assessed by Trypan blue staining was approximately 95%. This method has the advantage of simplicity, does not expose cells to any antigens, enzymes or antisera and does not compromise cell viability to a significant extent. With some modification of the type of column and serum used, and of the incubation procedure this methodology was applied in the separation of lymphocyte populations obtained by Ficoll-Hypaque centrifugation in the studies to be described later.

Measurement of Lymphocyte Activation:

Having obtained preparations of T and B lymphocytes it is necessary to review how the activation or suppression of B cells may be induced, and how this response may be measured. Three distinct levels of B cell maturation may be considered.

(1) Precursor B cells - cells which will eventually differentiate into mature B cells but early on are devoid of surface Ig, although they contain IgM in their cytoplasm[196].

(2) Resting B cells - small diameter lymphocytes having Ig on their surface, capable of synthesizing Ig molecules and displaying them as antigen binding receptors on the plasma membrane[197].

(3) Activated B cells - large lymphoblasts or plasma cells that develop after activation of (2). Increased protein synthesis indicated electron-microscopically by well developed rough endoplasmic reticulum, polyribosomes and Golgi apparatus leading to the production and transport of large quantities of immunoglobulin[198].

It is the activation of resting B lymphocytes which has received most attention in the last few years. Activation may result in end points (cell division, Ig synthesis or both[199, 200, 201]) which can be measured by several available techniques. It has been noted that proliferation does not necessarily result in increased Ig synthesis, and conversely that immunoglobulin synthesis is not always preceded by cell division[201]. The incorporation of ^{3}H-thymidine into DNA has been widely used as a measure of cell proliferation.

Immunoglobulin synthesis may be measured in one of three ways:

(1) Enumeration of Ig secreting cells.

(2) Immunochemical measurement of secreted Ig.

(3) Assays for antigen specific antibody.

(1) Enumeration of Ig secreting cells - Immunofluorescent staining has been the most widely used method of intracytoplasmic measurement of the number of differentiated B cells. It has a high specificity and sensitivity[202, 203] and can be performed with relative ease. The reverse hemolytic plaque assay (RHPA) which will be described in detail later, while being technically more difficult and expensive in time and reagents, is also very sensitive and falls into this category. Finally, electron-microscopy has been used (as already noted) as an indicator of B cell activation. These tests measure the number of activated

B cells and not the rate or total amount of immunoglobulin produced.

(2) Immunochemical measurement of secreted immunoglobulin - These assays have been developed to measure Ig which has been secreted. They are of two types:

(a) Using a competitive radio-immuno-assay, Ig secreted during the course of culturing is measured (at the end of the culture period)[204]. These assays are sensitive but use significant amounts of radio-isotope, require frequent calibration and significant levels of polyclonal B cell activation[205,206].

(b) Ig synthesized de novo can be measured using radio-labelled amino acid precursors. The resulting radio labelled Ig is then either precipitated[207] or adsorbed[208].

(3) Assays for antigen specific antibody - Recently a number of assays have been developed which measure antigen specific antibody on B cells or in culture supernatants. Plaque forming assays measuring cells secreting antibody against sheep red blood cells following polyclonal activation of B cells by pokeweed mitogen[209], allogeneic interaction[210] and helper

factors[211] have been easier to develop than those measuring responses to specific protein antigens such as ovalbumin[212] or TNP-modified red cells[213]. Soluble phase and solid phase sandwich radio-immune-assays have been used to measure antigen specific antibody in supernatants[214].

Polyclonal B Cell Activators (PBA):

Human B lymphocytes may be activated using a number of different methods. Three plant lectins phytohemogglutinin (PHA), conconavalin A (Con A) and pokeweed mitogen (PWM) whilst binding to the surface of B lymphocytes do not directly produce polyclonal B cell activation. Although these lectins bind to the surface of B cells they require the presence of T cells to produce activation, i.e. simple binding to B cells does not result in activation. This dependance on T cells has been demonstrated in B cell cultures completely depleted of T cells[215]. However, it should be noted that as little as 1% contamination with T cells is sufficient to allow the response to proceed in an otherwise pure B cell culture.

Several bacterial products have been recognised to be polyclonal B cell activators (PBA) - killed staphylococcus aureus, staphylococcal A protein, lipopolysaccharide, nocardia water-soluble antigen and purified protein derivative. Some

fetal calf sera (FCS) produce a high background activation noise presumably because of their content of unidentified B cell mitogens[216]. It is important therefore when using FCS in activation studies to use heat-inactivated sheep red blood cell absorbed FCS, to standardize procedure and establish normal controls.

Antisera to human β2 microglobulin (raised in rabbits), anti-HLA allo-antisera and antisera to surface IgM have been variably reported as mitogenic for B cells, but reports from different laboratories have been conflicting.

T lymphocytes may respond in a specific or non specific manner. Non antigen specific and antigen specific helper factors have been harvested from supernatants of both murine and human cell cultures. Lymphocyte mitogenic factor (LMF), an antigen-induced T helper factor has been recognized in human culture systems. It has been reported that while the release of LMF is antigen specific (i.e. released only from "educated" T cells), its action is nonspecific, and furthermore can cross allogeneic boundaries[216]. The nature of human LMF and its site of action on the B lymphocyte are not entirely clear. While it has the physiochemical properties

of a protein, immunosorbent column work (using columns containing antibodies to IgG and IgM heavy chains) suggests that LMF does not contain immunoglobulin chain determinants. LMF has been observed to increase proliferation of, oral Ig synthesis from, human B lymphocytes.

In contrast to nonspecific T cell helper factors, antigen-specific helper factors (ASF) induce B cells to produce antibody that is exclusively directed against the antigen used to elicit the T cell helper activity. The role of soluble helper factors in polyclonal B cell activation will be discussed further in a later chapter.

Manipulation of Subpopulations:

A review of the literature so far, has identified methods for (a) separating mononuclear cells from whole blood (Ficoll-Hypaque centrifugation), (b) for separating B and T lymphocytes (nylon wool column), (c) for activating lymphocytes (PWM) and (d) for measuring immunoglobulin synthesis (reverse hemolytic plaque assay). Prior to discussing plaque assays, several methods of manipulating lymphocytes in order to examine mechanisms which regulate the immune response will be reviewed.

Since B cell responses are an expression of the balance which exists between helper and suppressor influences, it is clear that changes in this balance may be effected by alteration in the number or activity of cells, having opposing actions. However, while PWM triggers T helper cells it also simultaneously triggers suppressor cells[218]. Therefore, it is necessary to selectively inhibit either help or suppression in order to measure the magnitude and direction of the other. Several methods of manipulation are available:

1. Radiation
2. Corticosteroids
3. Azothiaprine
4. cAMP
5. Mitomycin C.
6. Conconavalin A.

1. Radiation: - Previous studies have demonstrated that different lymphocyte subsets have differing radio-sensitivities. B cells are exquisitely sensitive and are functionally destroyed at very low radiation doses (200-300 rads). T helper cells are resistant to doses as high as 5,000-6,000 rads, while T suppressor cells have an intermediate sensitivity (1500-2000 rads). However, reports of the level of radio-

sensitivity have varied somewhat between laboratories doubtless reflecting differences in techniques and materials[219,220,221]. Nevertheless, relative radiosensitivities of subpopulations have been retained. The relative radiosensitivity of T suppressor cells allows a convenient method of adding functionally pure T helper cells to coculture of autologous or allogeneic B cells, merely by irraditing (at some optimal dose) the T cell suspensions prior to coculture. With this technique helper cell function of different T cell suspensions and the ability of B cells to respond appropriately to T helper cell influences can be assessed. (A further use for irradiation of T cell suspensions, is to eliminate by low dose radiation, B cells that have been carried over when T cells have been fractionated by E rosette enrichment).

2. Corticosteroids: - It has been noted that the addition of corticosteroids directly to lymphocyte cultures, selectively inhibits T suppressor cell function while relatively sparing T helper function, and thereby enhances the PWM driven anti-SRBC PFC response[222]. In contrast an effect has not been noted, with the acute in vivo administration of corticosteroids prior to venepuncture, although

Saxon et al[223] (using a different system) demonstrated a suppressor effect on B cells and T suppressor cells, with no effect on T helper cells.

3. Azothiaprine: - The effect of Axothiaprine is similar to that of irradiation i.e. B cells are very sensitive, T suppressor cells less sensitive, and T helper cells relatively resistant[224]. Thus when T cells are treated with Azothiaprine prior to culture they enhance the B cell response.

4. cAMP: - Limited studies have been conducted with cyclic AMP and cyclic AMP inducing agents, but it would appear that cells are relatively resistant in vitro to cyclic AMP, T suppressor cells being less resistant than T helper cells[225].

5. Mitomycin C: - A net helper effect has been noted on treating T cells with this agent using a PFC assay.

6. Conconavalin A: (Con A) - When T cells are activated by optimal mitogenic concentrations of Con A they are capable of suppressing PWM induced B cell activation[226, 227,228,229]. Reinherz and Schlossman[230] have identified the Con A suppressor cells as belonging to a distinct subset of T cells comprising 20% of the total circulating T cell population.

Plaque Assays:

The hemolytic plaque technique was introduced by Jerne and Nordin[231] in 1963, and allowed visualisation of the presence of a minute amount of biological material by a magnification process restricted to a local area. They developed a technique for scoring individual antibody forming cells in a rabbit lymph node model. A rabbit was injected three times with sheep red cell stromata, a cell suspension being prepared three weeks later from a popliteal lymph node. When a proportion of these lymphoid cells were incubated in an agar solution with sheep red cells and then complement was added, clear circular areas of haemolysis (plaques) appeared in a field of closely scattered red cells. Thus lymphoid cells released lytic antibody and caused complement dependent lysis of adjacent sheep red cells. In the centre of each plaque a lymphoid cell (presumably that which released the synthesised antibody) could be recognized.

In 1974[232] the methodology and theory of this technique using murine lymphoid cells were reported in detail, although several variations had been developed in the interim. Some researchers experimented with nonagar gells[233] while others examined cells producing classes of antibody other than IgM[234,235]. Indicator red cells were coated to detect

responses to more complex antigens and methods of performing the plaque assay on microscope slides[236] rather than in Petri dishes developed. Some features of the plaque assay require particular attention:

(1) Lymphocyte preparations must be relatively free of autologous red cells or else the lysis of test red cells will be masked and plaque forming cells difficult to detect. Jerne using a murine system noted the importance of using indicator cells (sheep erythrocytes) that were neither too young (resistant to lysis) nor too old (too fragile).

(2) Since red cell lysis is complement dependent the choice of a gel, such as agarose, which is not anti-complimentary is important. When gel concentration in the murine model was raised above 1% there was a decrease in plaque size and plaque diameter.

(3) Sheep red cell concentration in the indicator lawn also affects plaque size. With lower concentrations, plaques tend to be larger and less well defined at the margin but unchanged in number. In Jerne's model optimal sheep red cell concentration was $2x10^8$ SRBC per ml.

(4) It has been noted that incubation at a higher ($45^{o}C$) temperature than normal ($37^{o}C$) does not affect the plaque response. (This observation has some importance since agarose is usually heated until it liquifies and then maintained in a water bath at a temperature just above $45^{o}C$ until needed for plaquing. Then while still liquid, small aliquots of agarose are mixed with sheep red blood cells and lymphocytes and subsequently plaqued; incubation being conducted at $37^{o}C$ allowing the gel to set. However, it is evident from these incubation experiments that the momentary exposure of cells to "hot" agarose during mixing, which might raise concern regarding cell viability, is most unlikely to adversely affect the plaque response.) In contrast, incubation at lower temperatures ($<37^{o}C$) results in a reduction of plaque numbers.

(5) True plaques require differentiation from background plaques and spurious plaques.

(a) True plaques are those appearing only after the addition of complement and containing an antibody releasing cell at their centre. The number of plaques obtained from the same sample must be proportional to the number of cells plated.

(b) Spleen cell preparations from animals which have never been exposed to sheep red cells contain a small number of plaque-forming cells against such red blood cells (background plaques). It is not known whether they arise spontaneously or whether they result from previous exposure to cross-reacting bacterial antigens. They are morphologically indistinguishable from true plaques.

(c) Plaques not fulfilling the criteria for (a) or (b) are termed spurious plaques. They may be of several types:

1. Faults or bubbles in the agarose which can be identified by the depression they cause in the agarose surface on viewing it obliquely.
2. Tissue fragments or debris which are recognized by a dark visible spot in the centre of the plaque after Benzidine staining.
3. Haemolytic toxins produced by bacteria in contaminated preparation may produce haemolysis which is independent of lymphoid cells or complement. Benzidine staining may reveal a bacterial colony in the centre of the plaque.
4. Late plaques are those which occur with prolonged (greater than 24 hours incubation and which are likely related to the release of lysin from degenerate white cells.
5. Auto-haemolytic antibody forming cells from sheep. Rarely a batch of sheep red cells contains plaque forming elements which produce areas of haemolysis independent of test lymphocytes.

The active metabolism of plaque-forming cells has been demonstrated by ^{3}H-thymidine incorporation, and by the inhibition of plaque formation on lowering incubation temperature, or the addition of potassium cyanide, puromycin or cycloheximide.

While this direct technique has been used with success in systems using high efficiency haemolytic antibody, there are situations in which cells produce low efficiency haemolytic antibody and have difficulty in producing plaques. It is in the latter situation that indirect techniques have proven useful. One in particular requires the addition of anti-Ig (polyspecific or class specific) prior to incubation with complement. The anti-Ig molecules attach to those antibody molecules which have become fixed to the indicator red cells during the first incubation. In the presence of these complexes on the red cell surface complement mediated lysis is facilitated. The concentration of antiserum is important since high concentrations inhibit plaque formation, (both the direct plaques which may have occurred and the indirect plaques which should have occurred), whilst low concentrations result in poor plaque development. If these two methods (direct and indirect) are performed simultaneously, the difference between the two provides a measure of the number of facilitated plaques. Using different concentrations of anti-Ig (facilitating serum) the maximal response and therefore the optimal anti-Ig concentration can be found. Several methods have been devised to distinguish IgM and IgG plaques[232, 235, 237].

Jerne's original method was conducted on petri dishes, however a single layer technique conducted on microscope slides was devised by Mishell and Dutton[236]. The method involved coating microscope slides with 0.1 to 0.2% agarose and allowing them to dry. Sheep red cells and lymphoid cells were added to 0.5 mls of agarose (45°C) and poured on to the coated slide. Incubation was conducted on Lucite racks provided with a shallow (0.1 cm) well. For counting, slides were first dipped in saline and then their backs wiped dry. They were then viewed in bright indirect light. 40-100 plaques/slide were considered optimal, although it was possible to accurately count up to 200. The advantage of the slide method is that it only requires small quantities of facilitating serum, complement and indicator cells, and is economical when replicate determinations are being performed.

So far the target described has been a red blood cell; procedures scoring cells making anti-red cell antibodies. With some modification this technique can be applied to the detection of cells producing antibody against other antigens (proteins, polypeptides, polysaccharides and haptens). This is achieved by coating the indicator red cells, a process which must result in an adequate density of antigenic determinants, without causing increased red cell fragility, or

decreased sensitivity to complement mediated lysis. The conjugates should not by themselves lyse in the presence of complement. If cell suspensions are assayed on coated cells, a duplicate series on uncoated cells should be used, to determine by subtraction the background count.

Possibly the simplest chemical method of coating red cells with protein, is that using chromium chloride. Several authors have reported the coupling procedure using different types of red cells, proteins and methods[238,244]. The procedure basically involves the addition of given proportions of coupling protein to washed red cells, then introducing prepared chromium chloride into the mixture and mechanically agitating the resulting suspension for a given period of time. Cells are then washed to get rid of excess chromium chloride and unbound protein. The procedure has usually been performed at room temperature with a 1:1 cell protein volume ratio. In one study chromium chloride was added to protein[238] rather than to the mixture and in two studies the mixture was not agitated[238, 240]. There was some concern in early reports[239,241] that the number of plaques detected, especially in gel assays, (when cells had been coupled with chromium chloride) was rather low.

Coding[242] reviewing some aspects of the procedure, noted that the volume, concentration and age of chromium chloride, the concentration of coating protein and the adequacy of mixing affected the efficiency of coupling. A 1 ml volume of 0.01% concentration of aged (greater than 8 weeks) chromium chloride was found optimal. In addition he noted that the optimal amount of chromium chloride, was that which was just below the amount necessary to cause agglutination, and that chromium chloride was best added dropwise with constant mixing to ensure even coupling. Furthermore, he emphasized that chromium chloride should be added last, since it is rapidly inactivated by protein. With attention to these parameters the method is fast, efficient and many of the problems experienced by earlier workers overcome. Kofler and Wick[243] drew attention to the importance of controlling the pH of solutions around 5.0 in their system. Coupling failed when the pH fell below 5.0. The exact nature of the coupling mechanism is unknown, although it is believed that chromium chloride activates carboxyl residues on proteins in the RBC membrane, thereby providing active sites for binding antigens.

In examining data from plaque assays a number of potential sources of variation should be considered:

1. Biological variation in the blood donor.
2. Variation in plaque numbers when plaquing duplicate cultures from the same individual on the same day.
3. Variation in plaque numbers when plaquing the same cell suspension in duplicate.
4. Methodologic variations from day to day.

While variation cannot be completely overcome it can be reduced by replicate determinations and its effect in part accounted for by running simultaneous controls. Jerne[232] evaluated some of these sources of variation and incorporated them in his plaque theory. However, his model differed from the one chosen for the studies to be described later and therefore cannot be directly applied. Nevertheless, this early work in the development of plaque assay techniques and in coupling proteins to indicator red cells formed the basis on which more sophisticated assays were developed.

The major limitation of the previously described plaque assay was that it could only detect cells producing specific antibody directed against a known antigen. In 1974 Molinaro and Dray[245] separated lymphocytes from human blood by Ficoll Hypaque centrifugation and prepared rabbit anti-human IgG

which they coupled (by chromium chloride) to sheep red blood cells (Ab-E). The potential for a new type of plaque assay was realized. In the following year Eby et al[246] described the reverse haemolytic plaque assay (RHPA), in which indicator sheep erythrocytes were coated with anti-human immunoglobulin antibody. This technique allowed detection of individual human immunoglobulin secreting cells irrespective of the antigen specificity of the antibody secreted. Following incubation of human lymphocytes, Ab-E and agarose, the resulting gel was incubated in a petri dish in a humidified environment for one hour, then in developing serum (anti-human Ig serum) for one hour, and finally in guinea pig complement for one hour. The important findings of this study[246] were:-

(a) At least 1% of human mononuclear cells secreted immunoglobulin.
(b) 1.16 $\pm$ 0.4% of plated cells formed plaques.
(c) 1 mm^3 of whole blood contained 22 $\pm$ 8 Ig secreting cells.
(d) Lymphocytes not treated with facilitating serum and with complement failed to develop plaques.
(e) Lymphocytes plated with goat anti-rabbit Ig Ab-E or with rabbit anti-human Ig Ab-E together with soluble anti-human Ig antibody formed few plaques, indicating specificity (mediated by Ig molecules) of plaque formation.

(f) On the basis of several determinations, the reproducability of the assay was shown to be adequate when (i) lymphocytes were treated with separate preparations of Ab-E and when (ii) different samples of reference lymphocytes were tested on preparations of Ab-E prepared on the same day. It was noted, however, that different preparations of anti-human Ig antibodies from different rabbits produced different numbers of plaques.

(g) The response in 10 normals was 6,100 - 17,600 PFC/10^6 mononuclear cells.

(h) The narrow range of percentages obtained for percent plaque-forming cells in normals suggested a homeostatic mechanism.

(i) Using different washing and incubation procedures the possibility that PFC formation was due to released membrane-bound Ig monocytes, or due to simple release rather than active secretion from B cells was excluded.

Thus the Reverse Haemolytic Plaque Assay depends on active secretion of immunoglobulin from viable cells[232,246] and has several advantages over previously described systems. Fluorescent anti-Ig antibody techniques detect cells which bear or contain Ig but do not specifically identify those cells which secrete it, while quantitation procedures measure total Ig in lymphocyte cultures but do not allow assessment of the secretory activity of individual lymphocytes. Ginsburg[247] studied the

kinetics of the PFC response using a modified RHPA and made the following important observations in 15 normal individuals:

(a) The plaque response was negligible when mononuclear cells were plaqued without culturing.
(b) The peak response was obtained when mononuclear cells were cultured for 5 to 7 days prior to plaquing.
(c) Cells cultured longer for 7 days showed a decline in their response.
(d) Culture of mononuclear cells with pokeweed mitogen prior to plaquing resulted in a tenfold greater PFC response.
(e) PWM increased the proportion of IgM secreting cells in cultures.
(f) The dependence of the response on active secretion of immunoglobulin was confirmed.
(g) 0.1% of circulating mononuclear cells actively secreted immunoglobulin, a value somewhat lower than that calculated by Eby. This difference may reflect variation in the sensitivities of the two systems.
(h) Incomplete washing of mononuclear cells prior to plaquing resulted in the carry over of sufficient immunoglobulin to produce pseudo plaques or complete lysis of red cells in the indicator lawn, highlighting the importance of adequate cell washing.

(i) There were in most cultures a small percentage of cells simultaneously secreting more than 1 Ig isotype.

In studies 1-5, to be described in the next section, the reverse haemolytic plaque forming cell assay was used as the indicator technique by which the net effects of the help/suppression balance on Ig synthesis by B cells would be measured.

As is common to many biologic systems, there has been some variation in the results obtained when the same end-points have been measured using different techniques, and when the same end points have been measured by the same techniques but in different laboratories. For this reason it was necessary to validate and standardize the aforementioned techniques in London, prior to applying them in a formal comparison of patients with rheumatoid arthritis and normal subjects.

STANDARDISATION EXPERIMENTS

- INTRODUCTION

- MATERIALS AND METHODS

- STUDY 1. Effect of culture duration on PFC response (i)
  2. Effect of culture duration on PFC response (ii)
  3. Description of pokeweed mitogen dose/response curve.
  4. Comparison of three sources of foetal calf serum.
  5. Description of radiation dose/response curve for T lymphocytes.

- SUMMARY

INTRODUCTION.

Culture duration, pokeweed mitogen (PWM) concentration, origin of fetal calf serum (FCS) and radiation dose are factors known to affect immunoglobulin synthesis by B lymphocytes in the system to be described. It was the intention with these early studies to gain experience with techniques, identify methodologic problems, describe dose response effects and choose optimal conditions for subsequent studies. The experimental method is outlined schematically in Figure 14.

Only patients with ARA criteria for classic or definite rheumatoid arthritis (Tables 1-3) were selected for study. Normal subjects were chosen as controls. Patients with rheumatoid arthritis are identified by capital letters and their profiles displayed in Appendix 1, while controls are identified by small letters and their profiles shown in Appendix 2. Original data are tabulated in Appendix 3. (Subject identification, culture number, cell concentration and combination, the presence or absence of pokeweed mitogen, the radiation dose for T cells if radiated, the culture duration, plaque number and response expressed as PFC/10^6 PBL are tabulated). In some instances cell combinations were cultured singly and plaqued once, in others they were cultured in

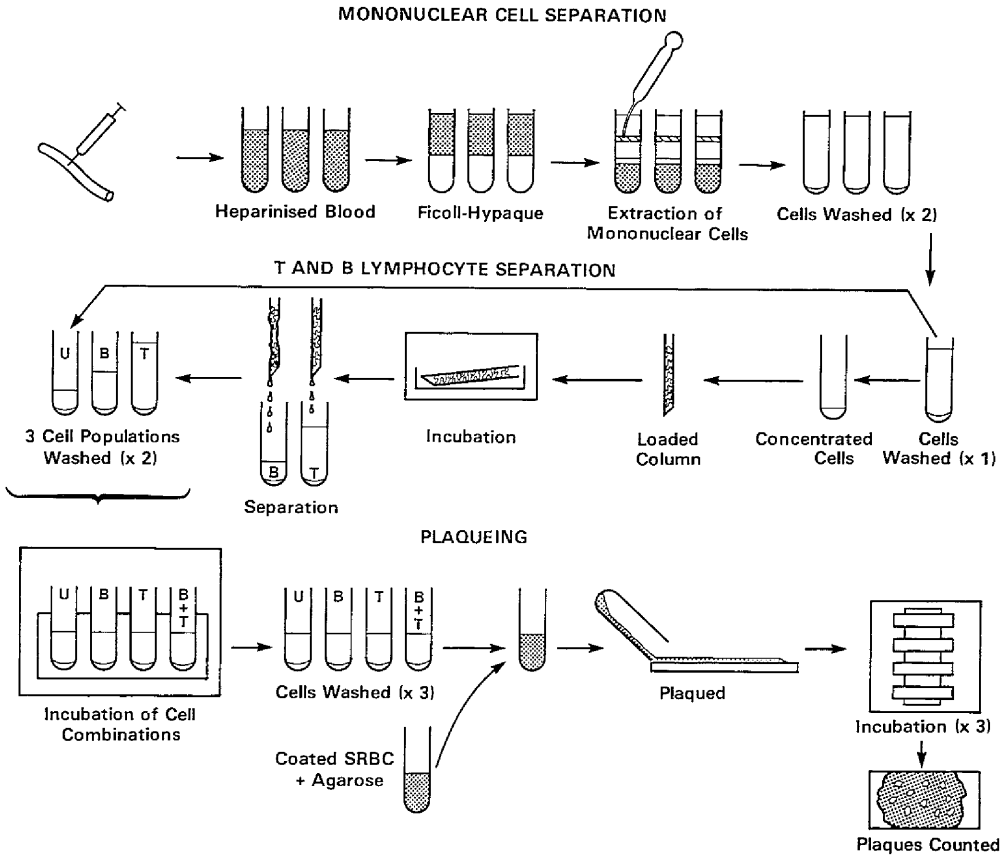


FIGURE 14 - SCHEMATIC REPRESENTATION OF STANDARD PROCEDURE.

U = Unseparated lymphocytes; B = B lymphocytes; T = T lymphocytes; SRBC = Sheep Red Blood Cells.

duplicate and each replicate plaqued once, and in yet another they were cultured in duplicate and each replicate plaqued twice. The results expressed in the following tables are the means and standard error of the mean calculated from between 1 and 4 data points.

MATERIALS AND METHODS.

Subjects:

Three patients who fulfilled ARA criteria for classic or definite rheumatoid arthritis, and four normal subjects were chosen for studies 1-5.

Cell separation:

Although a sterile hood, gloves and mask were not used, the following procedures were conducted at the bench in as sterile a manner as possible in order to avoid contamination of the resulting cultures. The incubator was a potential source of contamination and was regularly cleaned.

Mononuclear cells were separated from 30 mls of fresh heparinized blood by Ficoll Hypaque (Ficoll 400, Pharmacia Fine Chemicals AB Uppsala, Sweden) (Hypaque, Winthrop Laboratories, Aurora, Ontario) (SG 1.079-1.084) centrifugation. Centrifugation rates are described in centrifugal force (g) rather than RPM since the length of rotor arm and centrifuge tube result in different g forces for a given rotor speed. Gravitational forces (g) quoted are measured at the bottom of the tube and can be translated into RPM using an International Centrifuges Normograph (International Equipment Limited, 300 Second Avenue, Needham Heights, Massachusetts, U.S.A. 02194). 10 ml aliquots of blood

were carefully layered with a Pasteur pipette on to 3.5 mls of Ficoll Hypaque contained in each of three plastic centrifuge tubes. (by holding the pipette tip against the side of the tube approximately 10 mm above the fluid level, and allowing blood to flow slowly downwards onto the surface of the Ficoll-Hypaque) Following centrifugation at 800 g for 15 minutes at $4^{o}C$ five layers could be observed. The bottom layer contained red blood cells and the thin second layer granulocytes. The third layer contained Ficoll-Hypaque and some mononuclear cells. The fourth layer was a narrow band of mononuclear cells, and the top layer contained mainly plasma. Cells were removed from the mononuclear layer by introducing a Pasteur pipette into the fourth level and releasing the bulb while simultaneously moving the tip in a clockwise direction within the narrow band to collect as many cells as possible. This procedure was repeated until all visible cells had been removed from this layer. Following extraction of mononuclear cells the Ficoll-Hypaque layer was also removed by introducing a pipette to the third level, tipping the tube slightly to one side and releasing the bulb. Care was taken not to pipette red blood cells or granulocytes. A significant proportion of the plasma layer

was also removed at the time of extracting mononuclear cells. Ficoll-Hypaque and the lower plasma layers were included in the collection since it was known that a small but significant percentage of mononuclear cells were present there. This procedure was repeated on the remaining two tubes in order to extract the maximum number of mononuclear cells from the original 30 mls of blood. Cells were washed three times in RPMI 1640, pH 7.4 (Grand Island Biological Co., Grand Island, New York 14072), the supernatant being removed with a vacuum pipette following each centrifugation (300 g for 8 minutes at 4°C), and the cells resuspended in RPMI 1640 using a Pasteur pipette. Following the second washing, cells from the three tubes were transferred by Pasteur pipette into a single tube, suspended in 5 mls of RPMI 1640 and a 0.5 ml aliquot removed and retained for studies on unseparated lymphocytes. Following the third centrifugation cells were resuspended in 0.5 ml of 5% fetal calf serum (Grand Island Biological Co., New York 14072) in RPMI 1640 at 37°C.

Adherent (B) and nonadherent (T) cells were separated using a modification of the nylon wool technique described by Werner et al[195]. Columns were prepared from 15 cm by 6 mm plastic drinking straws (Dover Industries, Hamilton, Ontario), one end being cut at 45° and heat sealed. 100 mg of nylon wool

were teased apart and packed evenly with a thin wooden rod into each straw, a minute hole being cut in the bevelled end to allow drainage. It was important that columns were packed evenly otherwise air-pockets formed on priming, making them unusable. Furthermore the hole cut at the bevelled end had to be large enough to allow slow steady drainage of drops of liquid, but not so large that a continuous torrent of liquid was delivered -- a factor which seemed to have an adverse effect on cell separation. Columns were rinsed through with 10 ml of RPMI 1640 and then 3 ml of 5% v/v fetal calf serum (Grand Island Biological Co., New York 14072) introduced with a Pasteur pipette into the open end of the vertically held column, following which they were placed on a slight inclination (to prevent drainage from the upper end), in a 37°C incubator for 30 minutes. Care was taken to ensure that the fluid level in the column was above the upper level of the nylon wool and that no air bubbles were present, thus avoiding drying of the wool during incubation. Peripheral blood mononuclear cells in 0.5 ml of 5% fetal calf serum in RPMI 1640 at 37°C were loaded on to each column with a Pasteur pipette whilst holding the column vertically. A small quantity of warmed (37°C) 5% fetal calf serum in RPMI 1640 was introduced into the open end to prevent drying. Columns were incubated in

an almost horizontal position for half an hour at 37°C. Non-adherent (B) cells were removed by running 10 mls of pre-warmed (37°C) 5% fetal calf serum in RPMI 1640 through the column using a Pasteur pipette, whilst holding it vertically over a plastic receiving tube. Adherent (B) cells were removed by rinsing the column with 3 x 1.5 ml of warm 5% fetal calf serum in RPMI 1640 while gently massaging the tube. There was found to be an optimal pressure for massaging the tube which would dislodge adherent cells without damaging them. The cells were thereafter washed twice in RPMI 1640 and resuspended following each centrifugation at 300 g for 8 minutes at 4°C. Adherent (B) cells were suspended in a final volume of 1 ml of RPMI 1640 and non-adherent (T) cells in 5 mls of RPMI 1640. (Ninety-eight per cent of non-adherent cells rosetted with sheep red blood cells, whereas less than 1% of adherent cells rosetted, indicating effective separation of B and T lymphocytes). At this point three populations of cells had been prepared - (1) unseparated lymphocytes, (2) non-adherent (T) cells and (3) adherent (B) cells. (In Study No. 5 0.5ml aliquots of T lymphocytes in RPMI 1640 were irradiated at one of several dosages between 800 and 6,400 rads. Cells were then washed three times in RPMI 1640 to free the

cells of contaminated extracellular debris, and resuspended to their original volumes following centrifugation at 300g for 8 minutes at $4^{o}C$.

Cell viability was assessed using a Trypan blue staining method and was found to be better than 98%. Viable cells excluded the stain whilst non-viable cells took on a blue appearance. Lymphocytes were counted (recognized by size and morphology) on a Neubauer counting chamber under a light microscope. Cell concentrations were determined for each cell population and the volume of cell suspensions required for culturing a known number of cells ($5x10^4$ or 10^5) calculated.

In separate studies mononuclear cells were cultured in replicate either as irradiated or non-irradiated T cells alone ($5x10^4$ or 10^5), or as B cells alone ($5x10^4$ or 10^5), or in a 1:1 ratio combination of $5x10^4$ irradiated or non-irradiated T cells with $5x10^4$ B cells, or as 10^5 unseparated mononuclear cells. Cells were cultured from 0 - 10 days at $37^{o}C$ in 1 ml of RPMI 1640 (containing L glutamine) supplemented with 10% heat inactivated sheep red blood cell absorbed fetal calf serum (from 1 of 3 sources), and gentamicin 50 mcg/ml (Schering Corporation, Kenilworth, N.J. 07033) both with and without varying concentrations of pokeweed mitogen (PWM-Grand Island Biological Co., New York). Cultures were contained within

12 x 75 mm plastic tubes (Falcon Plastic, Cockeysville, Maryland) and maintained at 37°C in a humidified environment containing 5% CO_2. Following incubation cells were washed three times in RPMI 1640 and resuspended in 0.5 ml BSS pH 7.4 following centrifugation (300 g for 8 minutes at 4°C).

Quantitation of Immunoglobulin Synthesis (Figure 15)

Immunoglobulin synthesis by unseparated cells, B cells or various combinations of B and T cells were measured using a reverse haemolytic plaque forming cell assay employing rabbit polyspecific antiserum to human IgG, A and M isotypes (Cappel Laboratories, Cochraneville, Pa.) coupled to sheep erythrocytes (Woodlyn Laboratories Ltd., Guelph, Ontario) by aged (greater than 6 months) chromium chloride. The method used for coupling was similar to that described by Goding. Packed sheep red blood cells were washed three times in saline and finally resuspended in 0.5 mls sterile BSS following centrifugation (800 g for 5 minutes at 4°C). 1.5ml of 0.01% aged (greater than 6 months) chromium chloride were added dropwise with constant agitation to tubes containing 5ml of 0.9% saline, 0.5 ml absorbed polyspecific antihuman immunoglobulin (rabbit antiserum was adjusted to 2 mg of protein per ml by the method of Lowry) and 0.25 ml of washed packed sheep red blood cells.

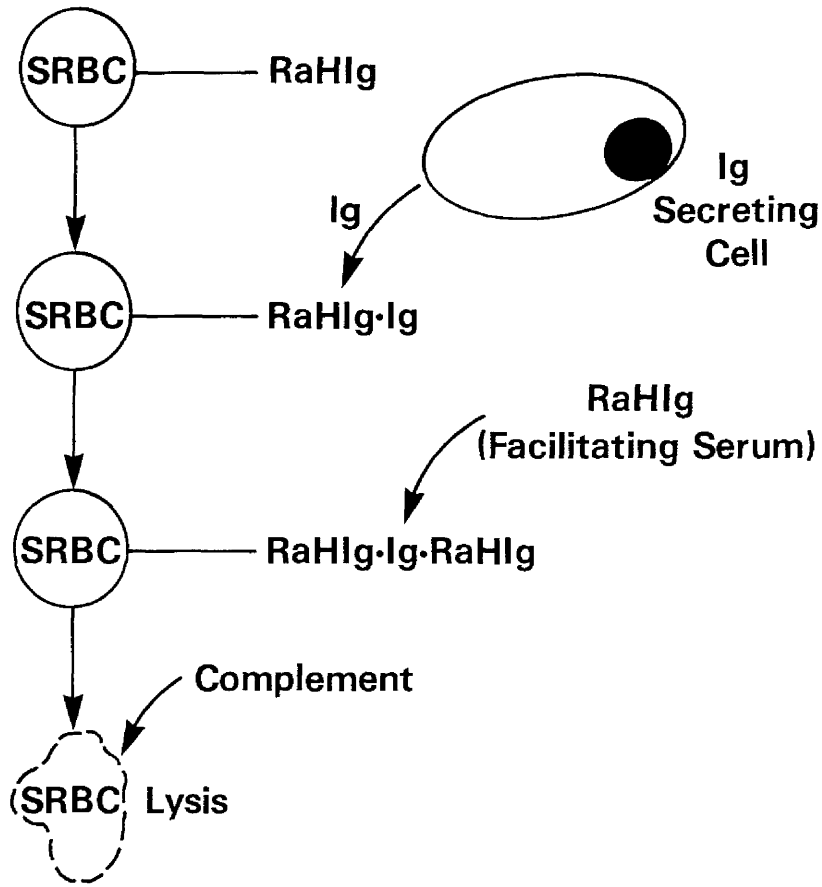


FIGURE 15. - REVERSE HAEMOLYTIC PLAQUE FORMING CELL ASSAY (RHPFC) PROCEDURE.
SRBC = Sheep Red Blood Cell. RaHIg = Rabbit antihuman immunoglobulin.
Ig = Immunoglobulin.

Agitation was continued for 5 minutes at room temperature, following which cells were washed three times in RPMI 1640, centrifuged (800 g for 5 minutes at 4°C), the supernatant discarded and finally resuspended in a ratio of 1 to 15 with BSS. Coupling was verified by passive hemaglutination using human Cohn Fraction II (Becton Dickinson, Orangeburg, N.Y.).

Cultures harvested between days 0 and 10 were infrequently contaminated (less than 1 in 300), in which event they were discarded. Contamination was recognized by marked discolouration or gelling of the culture medium, or the growth of mould on the surface. Cells were washed three times in RPMI 1640 and centrifuged at 800 g for 5 minutes at 4°C between each washing. Cells were finally resuspended in 1 ml of RPMI 1640. 100 microlitres of this cell suspension were plaqued with 50 microlitres of coupled sheep red blood cells in 0.5 ml of agarose on agarose coated microscope slides. The plaquing procedure involved preheating agarose and maintaining 0.5 mls of it in small glass tubes at 45°C in a water bath. 100 microlitres of cell suspension and 50 microlitres of coupled sheep red blood cells were separately pipetted into each tube. The tubes were vortexed slowly to ensure complete mixing and the contents carefully poured on to previously numbered and prepared agarose coated microscope slides. (Agarose coating was achieved

by drawing a brush previously dipped in warm liquid agarose across the surface of the slide and drying it in air. This preparation had the advantage that poured cell suspensions were contained on the slide rather than running off the edge.) These preparations were incubated on trays at 37°C in moist heat for one and a half hours, and thereafter incubation was continued for two hours in 1% facilitating serum (rabbit polyspecific antiserum to human IgG, A and M in sterile BSS), then three hours in 10% SRBC absorbed guinea pig complement (Cedarlane Laboratories Limited, London, Canada). For these last two incubations slides were placed face down in long trays, which had raised edges to keep the preparations just clear of the bottom of the container, the trough then being flooded with the appropriate solution. Following incubation plaque forming cells were counted and expressed per 10^6 viable peripheral blood lymphocytes. (PFC/10^6 PBL) A true plaque(Fig. 16) was recognized as a circular, relatively clear area, in the agarose and represented a lymphocyte surrounded by a rim of haemolyzed sheep red blood cells. This required differentiation from air bubbles and from pseudo plaques. The latter were recognized as depressions in the surface of the agarose on looking obliquely. The number of viable peripheral blood lymphocytes in each culture tube was calculated as follows.

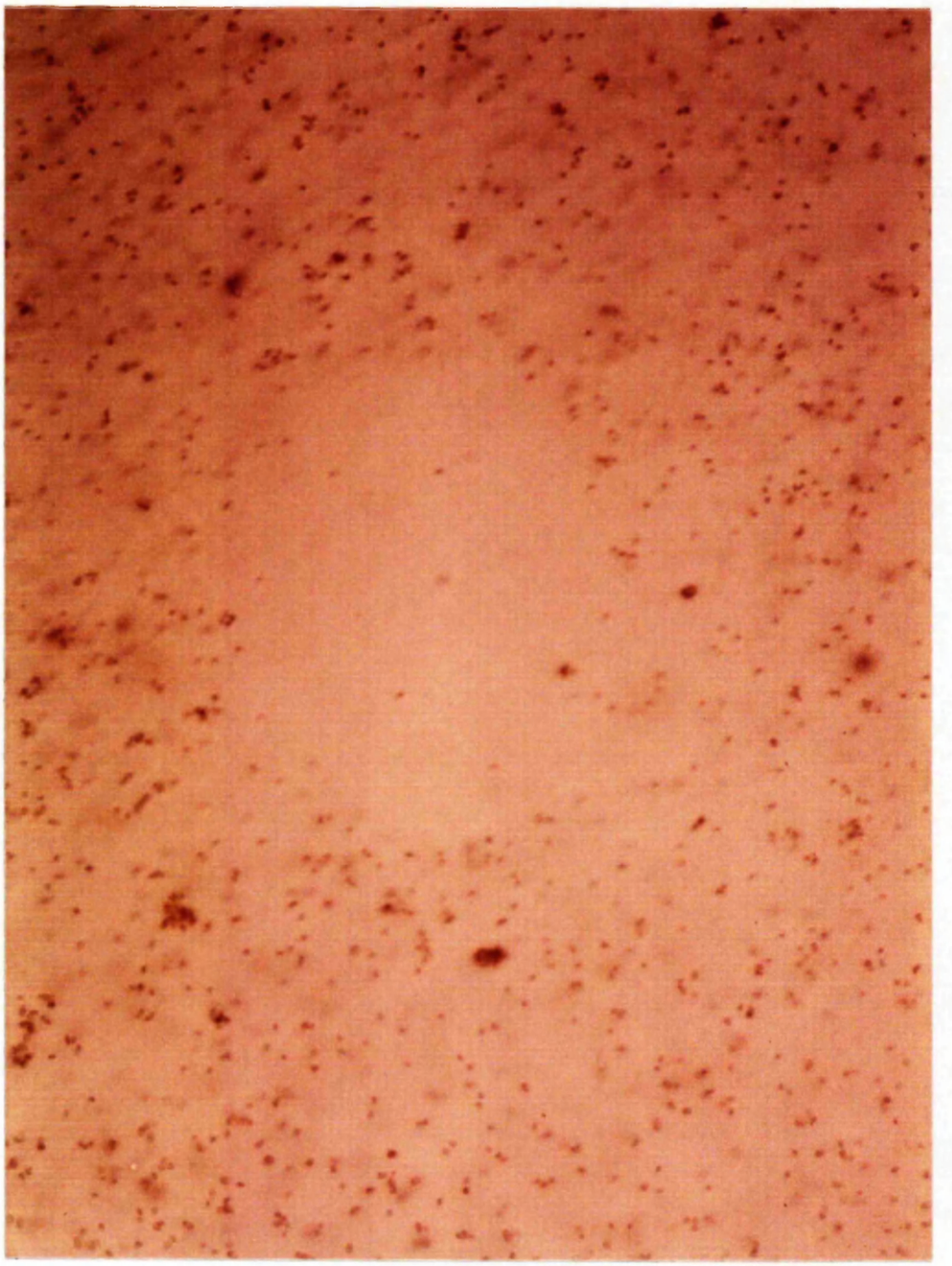


FIGURE 16 - TRUE PLAQUE.

A clear zone of haemolysis with a solitary central human peripheral blood B lymphocyte from a patient with rheumatoid arthritis can be observed in an indicator lawn of sheep red blood cells.

The total number of lymphocytes was counted by a machine, the accuracy being checked by manually counting selected samples. The viability was judged in 10 to 20 cultures by counting the number of healthy (cells identified as lymphocytes which had normal configuration and were not crenated or distorted) lymphocytes and expressing them as a percentage of the total number of lymphocytes. This percent viability was applied to all machine counted values. Results were expressed in this way since it was reasoned that non-viable lymphocytes would not contribute to the observed response. Thus following calculation of the number of viable lymphocytes in 100 µl of cell suspension, and knowing the number of plaque forming cells in that 100 µl, the number of PFC/10^6 PBL was easily calculated.

Studies 1 - 5:

In the 5 studies to be described conditions were varied as follows. In studies 1 and 2 culture duration was varied between 0 and 10 days in order to determine the culture duration which would provide the greatest response. In study 3 varying concentrations of pokeweed mitogen were used to define the shape of the dose response curve, and to determine the most appropriate concentration to use in later studies. In study 4 three different sources of fetal calf serum were compared in order to determine if any one was superior. In study 5 non-adherent (T) lymphocytes were irradiated at varying dosages prior to coculture with B cells in order to define the shape of the dose response curve and determine the relative radiosensitivities of T cell subpopulations. Any deviation from the general methods already described are defined in the conditions for each study.

Study No. 1. - Effects of varying culture duration.

Mononuclear cells from a single normal subject (e.c.) were separated and prepared as described above. The original data are shown in Appendix 3, cultures No. 1-60.

Conditions:

Four cell combinations were studied - 10^5 B cells alone, 10^5 T cells alone, 10^5 unseparated lymphocytes and 5×10^4 B cells with 5×10^4 T cells. Cells were cultured for 0, 3, 7 and 10 days with and without 1% PWM (Grand Island Biological Company, New York, Lot #15K0201). Heat inactivated sheep red blood cell absorbed fetal calf serum used in this study was from Grand Island Biological Company, Grand Island, New York. No cells were radiated. It has been observed that if too many cells were plaqued then the resulting plaques often coalesced making coupting either inaccurate or impossible. For this reason, in Study No. 1 rather than plaquing a fixed volume e.g. 100 microlitres, 10^5 cells were plaqued without culturing on day 0 when it was anticipated responses would be low, whereas $2x10^4$ - $5x10^4$ cells of each type were plaqued on days 3,7 and 10 when responses would be high. All responses (PFC) were finally referenced to 10^6 PBL.

Results:

In each instance responses were higher when cells were cultured with PWM than without. (Table 4) Immunoglobulin production was negligible on day 0 for all cell types. Thereafter responses increased progressively to be maximal at day 7 for all culture types except one (unseparated lymphocytes cultured without PWM). After day 7 responses invariably declined. The increment between days 0 and 3 was generally greater than that between days 3 and 7. Response was greatest for cultures containing B and T lymphocytes in the presence of PWM (3466 $\pm$ 278 PFC/10^6 PBL). B cells and unseparated lymphocytes with PWM also produced substantial responses. T cell responses were negligible. i.e. Nylon wool separation had been effective. Finally, the addition of T cells to B cells enhanced the response at 7 days, both with and without PWM.

Conclusion:

This study suggested the following:

1. The spontaneous plaque response was 0 and culturing of cells was necessary to produce the observed response. The finding provided the necessary evidence that immunoglobulin synthesis occurred during culture and that the responses seen were

TABLE 4. THE EFFECT OF CULTURE DURATION ON THE PFC RESPONSE IN A SINGLE NORMAL SUBJECT.

	IMMUNOGLOBULIN SYNTHESIS (PFC/10^6 PBL $\pm$ SEM)							
DAYS CULTURED	0		3		7		10	
PWM (1%)	+	-	+	-	+	-	+	-
CELL TYPE								
B	0	0	2230 $\pm$ 250	260 $\pm$ 60	2480 $\pm$ 240	570 $\pm$ 50	475 $\pm$ 25	325 $\pm$ 75
T	0	0	30 $\pm$ 10	30 $\pm$ 10	106 $\pm$ 41	35 $\pm$ 17	46 $\pm$ 18	30 $\pm$ 10
B & T	10	5	1580 $\pm$ 250	335 $\pm$ 5	3466 $\pm$ 278	770 $\pm$ 30	1690 $\pm$ 110	350 $\pm$ 30
UNSEP	-	-	2593 $\pm$ 47	540 $\pm$ 40	2800 $\pm$ 800	360 $\pm$ 40	260 $\pm$ 40	110 $\pm$ 30

not the result of immunoglobulin present on B lymphocytes at the time of venepuncture.

2. It was apparent that the optimal time for culturing cells was 7 days and that functional capacity declined significantly with longer durations.

3. Low responses from T cell cultures indicated that there was little B cell contamination of T cell preparations. i.e. nylon wool separation had been effective.

4. Immunoglobulin production from B cells was increased when they were co-cultured with T cells indicating an interaction occurring between subsets of lymphocytes.

5. Responses were increased 4 to 5 fold in the presence of 1% pokeweed mitogen indicating an effect of this T cell dependant B cell mitogen.

Study No. 2. - Effects of varying culture duration.

In order to verify the observation in Study No. 1 that immunoglobulin synthesis was maximal following 7 days of culture, a similar study was designed using a different normal subject (c.s.). The original data are shown in Appendix 3, cultures No. 61-126.

Conditions:

Four sets of cultures were established. 10^5 B cells alone, 10^5 T cells alone, $5x10^4$ B cells cocultured with $5x10^4$ T cells, and 10^5 unseparated cells. Cells were cultured for 0, 3, 7 and 10 days in the presence and the absence of 1% PWM (Grand Island Biological Company, Grand Island, New York, Lot #15K0201). Heat inactivated sheep red blood cell absorbed fetal calf serum was produced by Grand Island Biological Company, Grand Island, New York. No cells were radiated. 10^5 cells were plaqued on day 0 whereas $5x10^4$ cells were plaqued on days 3, 7 and 10 for the reasons previously outlined.

Results:

For all culture combinations responses were higher in the presence than in the absence of PWM (Table 5). T cell responses were minimal indicating adequate separation and

TABLE 5. THE EFFECT OF CULTURE DURATION ON THE PFC RESPONSE IN A SECOND NORMAL SUBJECT.

	IMMUNOGLOBULIN SYNTHESIS (PFC/10^6 PBL ± SEM)							
DAYS CULTURED	0		3		7		10	
PWM (1%)	+	-	+	-	+	-	+	-
CELL TYPE								
B	240±40	210 ± 10	1000 ± 250	300 ± 100	3425 ± 325	2025 ± 425	450 ± 50	100±50
T	40±20	30 ± 10	0	17 ± 17	600 ± 50	300 ± 100	0	25
B & T	390±10	360 ± 0	2138 ± 138	738 ± 188	4462 ± 338	838 ± 88	350 ± 50	250±50
UNSEP	410±10	400 ± 40	475 ± 75	500 ± 100	3150 ± 200	1125 ± 25	300 ± 100	125±75

little contamination of T cell preparations with B cells. Plaque responses on day 0 were minimal, although they were somewhat greater than in Study No. 1. Responses increased at day 3 and were maximal by day 7. Increments between day 3 and day 7 were generally greater than between day 0 and day 3. Invariably responses fell between day 7 and day 10. Responses were similar at day 10 and day 0. The maximum response ($4,462 \pm 338$ PFC/10^6 PBL) was obtained by coculturing B and T cells in the presence of PWM.

Conclusion:

This study verified the findings of Study No. 1.

1. Immunoglobulin synthesis was maximal on day 7 for all types of culture.
2. There was a decline in immunoglobulin synthesis after day 7 to a level at or about that found on day 0.
3. 1% pokeweed mitogen enhanced the observed response for all culture types.
4. The addition of T cells enhanced the B cell response, both in the presence and absence of PWM.

Combined results for Studies 1 and 2 are displayed in Table 6 and Figure 17. It was evident, that statements made independently in the first two studies regarding culture duration, cell separation and effects of PWM and T cell coculture held true when individuals were grouped. At 7 days under the conditions described the responses from cultures

TABLE 6. THE EFFECT OF CULTURE DURATION ON THE PFC RESPONSE - COMBINED RESULTS OF TWO NORMAL SUBJECTS.

DAYS CULTURED	IMMUNOGLOBULIN SYNTHESIS (PFC/10^6 PBL $\pm$ SEM)							
	0		3		7		10	
PWM (1%)	+	-	+	-	+	-	+	-
CELL TYPE								
B	120$\pm$120	105$\pm$105	1615$\pm$615	280$\pm$20	2953$\pm$473	1298$\pm$728	463$\pm$13	213$\pm$113
T	20$\pm$20	15$\pm$15	15$\pm$15	24$\pm$ 7	353$\pm$247	168$\pm$133	23$\pm$23	28$\pm$ 3
B & T	200$\pm$190	183$\pm$178	1859$\pm$279	537$\pm$202	3964$\pm$498	804$\pm$ 34	1020$\pm$670	300$\pm$ 50
UNSEP	410$\pm$10	400$\pm$40	1534$\pm$1059	520$\pm$20	2975$\pm$175	743$\pm$383	280$\pm$20	118$\pm$ 8

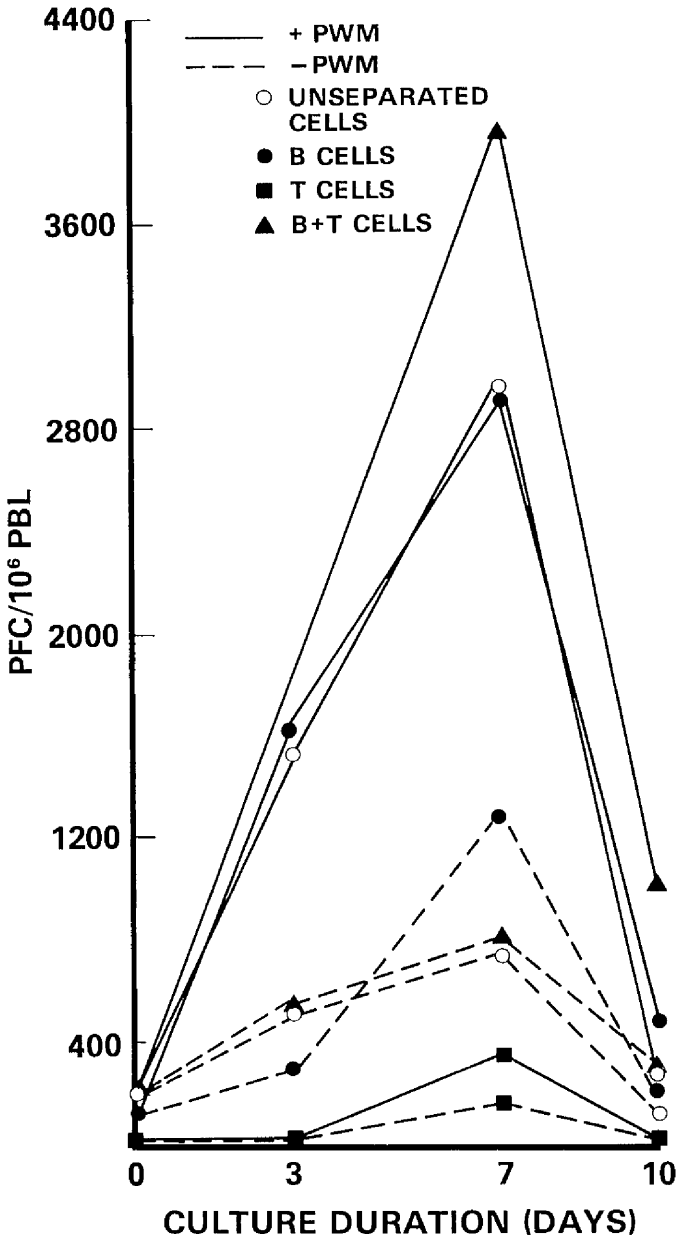


FIGURE 17. - EFFECT OF CULTURE DURATION ON PFC RESPONSES - COMBINED RESULTS FROM TWO NORMAL SUBJECTS.

containing PWM was 2 to 4 fold greater than those without. The mean B + PWM response was 2953 PFC/10^6 PBL which increased to 3964 PFC/10^6 PBL with the addition of T cells. Mean responses in cultures containing B + T cells in a 1:1 ratio (3964 PFC/10^6 PBL) were higher than those from unseparated lymphocytes (2975 PFC/10^6 PBL) where B:T cell ratios approximated those found in the peripheral blood of the subjects studied. Possibly a 1:1 B:T cell ratio favoured a higher response in this system.

It was on the basis of these two studies that 7 days was chosen as the standard time for culture duration in subsequent work. Furthermore, two sources of enhancement other than culture duration were identified, namely PWM and coculture with T cells.

Two modifications were made in the methodology hereafter. Firstly, since of the two types of lymphocytes only B cells produced immunoglobulin it seemed more appropriate to culture $5x10^4$ rather than 10^5 B cells alone, so that a better comparison could be drawn when the same number of B cells were cocultured with $5x10^4$ T cells. Similarly $5x10^4$ T cells were cultured alone rather than 10^5 cells. Unseparated lymphocytes continued to be

cultured at 10^5 so that culture concentrations were equivalent to those of B + T combinations. Secondly, instead of plaquing a known number of cells (10^5 on day 0 or $2x10^4$ to $5x10^4$ on days 3, 7 and 10) it was decided to plaque on day 7 a known volume (100 microlitres) of cell suspension and calculate its cell concentration afterwards. With this approach a manageable number of plaques which could be easily identified and accurately counted was achieved.

Study No. 3: - Definition of the PWM Response Curve.

The effect of varying PWM concentrations between 0 and 3% was evaluated in three normals (e.c., m.e., p.h.,) and one rheumatoid (S.W.). Original data are shown in Appendix 3, cultures No. 127-171.

Conditions;

Mononuclear cells were separated and prepared as previously described. Three sets of cultures were established: $5x10^4$ B cells alone, $5x10^4$ T cells alone and $5x10^4$ B cells with $5x10^4$ T cells. Fetal calf serum was of the same origin as in the studies No. 1 and 2. Although PWM was from a common source (Grand Island Biological Company) the Lot No. for studies on e.c. and S.W. was No. 15K0201 and the Lot No. for studies on m.e. and p.h. was 12N5302. Cultures containing $5x10^4$ B cells were cultured for 7 days under the conditions previously described. 100 microlitres of cell suspension were plaqued and the responses expressed as plaque forming cells per 10^6 peripheral blood lymphocytes. (PFC/10^6 PBL)

Results:

Effective cell separation was indicated by the negligible responses seen in cultures containing only T cells. (Table 7) In every instance the B cell response was enhanced by coculture with T cells. In 2 subjects (E.C., S.W.) in whom incomplete curves were constructed (Figure 18) a positive correlation was noted between plaque response and PWM concentration. Maximal responses (3466 and 4090 PFC/10^6 PBL) occurred at the highest concentration of PWM studies (1%). In the 2 subjects (m.e., p.h.) in whom complete curves were constructed, there was a marked increase in response when concentration was increased from 0 to 0.5%. Between 0.5% and 2% the curve was rounded and above 3% there was a marked decline in the response observed. The maximal response for M.E. (5050 PFC/10^6 PBL) was at 1.5%, and for p.h. (5425 PFC/10^6 PBL) at 1%. The shapes of these two curves indicated that there was less variation in the response when concentrations between 0.5% and 2% were used than when concentrations below and above this range were utilized.

Conclusions:

It was deduced from these studies that PWM concentration influenced the magnitude of the observed response. Suboptimal responses were noted with PWM concentrations which were both too low and too high. Taking into account the shape of the

TABLE 7. RELATIONSHIP BETWEEN POKEWEED MITOGEN (PWM) CONCENTRATION AND PFC RESPONSE IN R.A. AND NORMAL SUBJECTS.

SUBJECT	PWM (CONC^{n})	CELL TYPE		
		B	T	B & T
s.w.*	0	143	0	572
	0.01%	89	0	1073
	0.1%	159	0	2454
	1.0%	238	0	4090
e.c.*	0	570	35	770
	0.5%	934	50	1210
	1.0%	2480	106	3466
m.e.**	0	-	-	1275
	0.1%	-	-	2475
	0.5%	-	-	4275
	1.0%	-	-	4937
	1.5%	-	-	5050
	2.0%	-	-	5025
	3.0%	-	-	3562
p.h.**	0	-	-	750
	0.1%	-	-	2200
	0.5%	-	-	4412
	1.0%	-	-	5425
	1.5%	-	-	4700
	2.0%	-	-	4225
	3.0%	-	-	2500

* - PWM Lot No. 15K0201

** - PWM Lot No. 12N5302

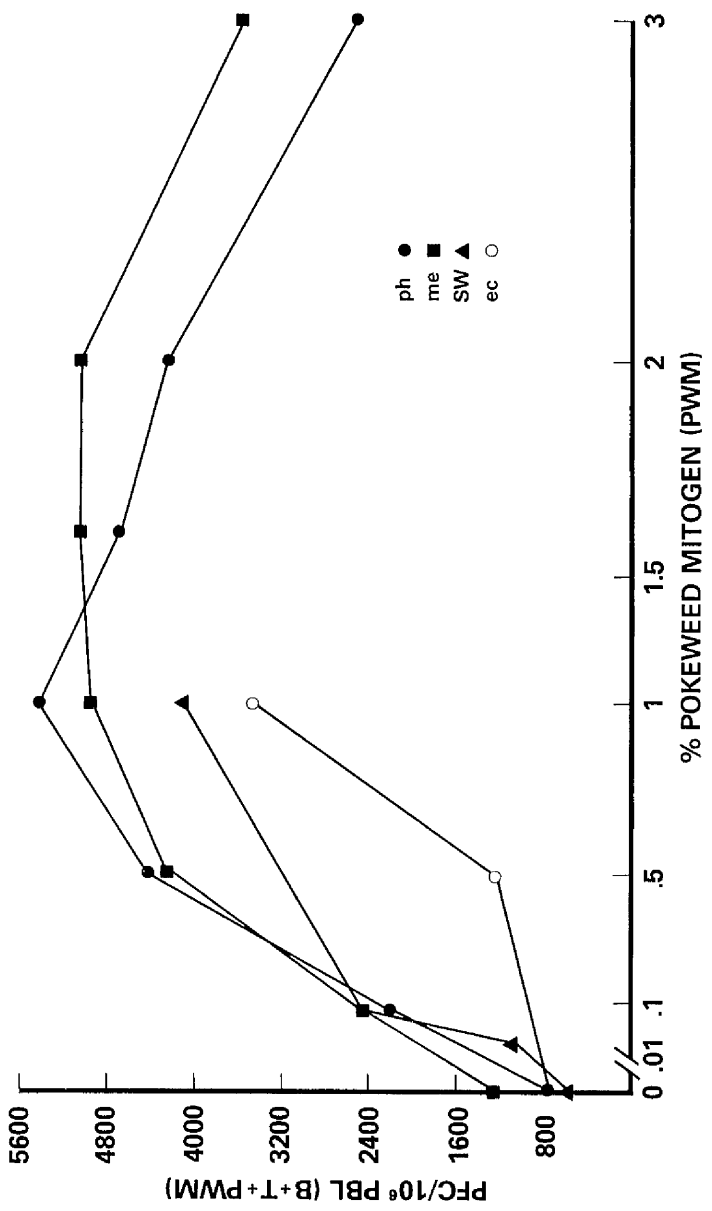


FIGURE 18.- DOSE/RESPONSE CURVES FOR POKEWEED MITOGEN - 3 NORMAL SUBJECTS (ph, me, and ec) AND A SINGLE RHEUMATOID (SW)

curve a standard concentration of 1% PWM was chosen for all subsequent studies. Furthermore it was evident that at the 1% concentration higher responses were achieved with PWM Lot No. 12N5302 than with 15K0201. For this reason PWM Lot numbers are quoted for each study and data from studies using different Lot numbers have not been pooled.

Study No. 4:- Evaluation of difference sources of fetal calf serum.

Fetal calf sera produced by Rehatuin, Flow and Gibco, were compared for efficacy in a single normal (e.c.) Original data are shown in Appendix 3, cultures No. 172-195.

Conditions:

Four sets of cultures were established containing $5x10^4$ B cells alone, $5x10^4$ T cells alone, 10^5 unseparated cells and $5x10^4$ B cells together with $5x10^4$ T cells. Cells were cultured for 7 days with and without 1% PWM (Gibco, Lot No. 15K0201). The experiment was triplicated, each of the 3 sources of fetal calf serum being used in turn. A constant source of fetal calf serum was used in cell separation, preparation and cell culture for each experiment. Concentrations were standardized and were as indicated in Materials and Methods. Each culture was subsequently plaqued in duplicate.

Results:

As had been previously observed, responses were invariably greater in the presence than in the absence of 1% PWM (Table 8).

TABLE 8. COMPARISON OF THREE SOURCES OF FOETAL CALF SERUM (FCS)

	IMMUNOGLOBULIN SYNTHESIS (PFC/10^6 PBL $\pm$ SEM)					
SOURCE OF FCS	REHATUIN		GIBCO		FLOW	
PWM (1%)	+	-	+	-	+	-
CELL TYPE						
B	475 $\pm$ 125	50 $\pm$ 50	958 $\pm$ 178	248	875 $\pm$ 250	249 $\pm$ 249
T	62 $\pm$ 62	0	62 $\pm$ 0	82 $\pm$ 28	250 $\pm$ 250	0
B & T	3640 $\pm$ 140	456 $\pm$ 42	4375 $\pm$ 875	426 $\pm$ 0	4140 $\pm$ 315	412 $\pm$ 83
UNSEP	4860 $\pm$ 45	53 $\pm$ 0	6889 $\pm$ 1660	92 $\pm$ 0	2883 $\pm$ 341	260 $\pm$ 20

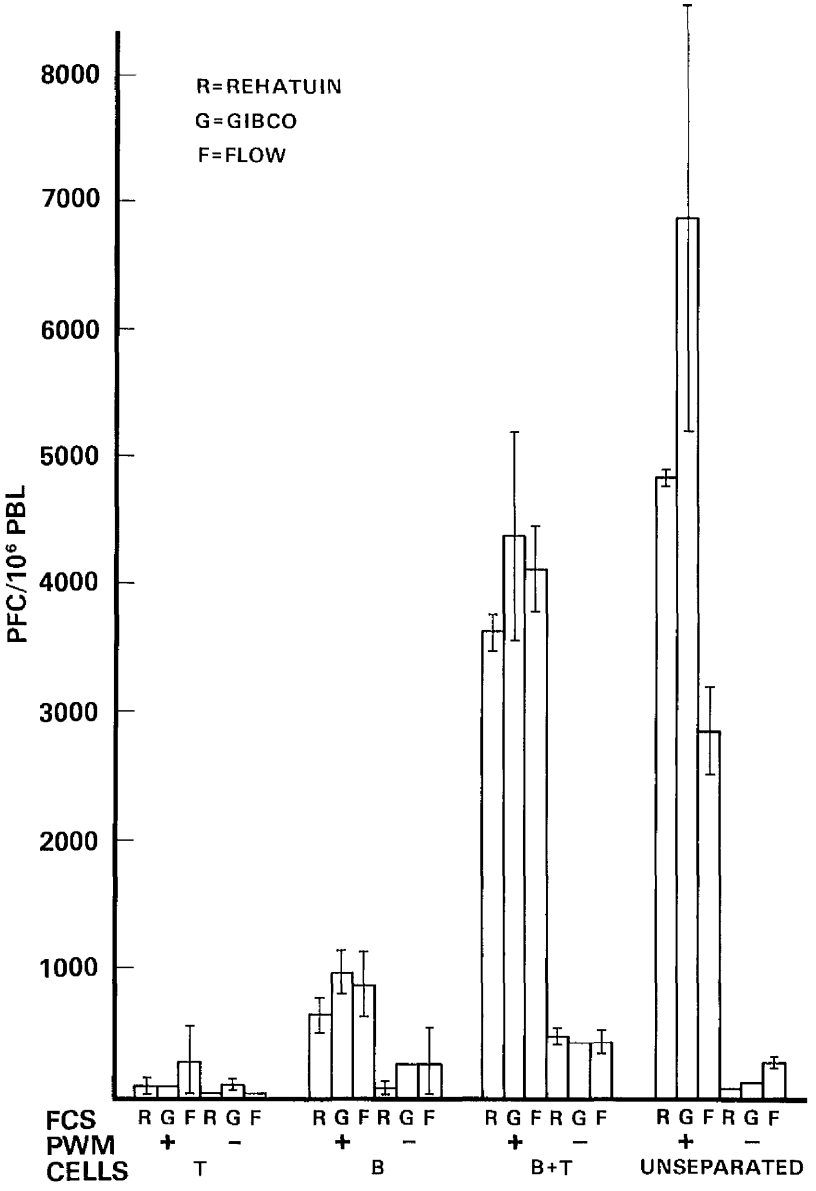


FIGURE 19 - PFC RESPONSES OF NORMAL LYMPHOCYTES - COMPARISON OF THREE SOURCES OF FOETAL CALF SERUM.

The greatest responses were observed with unseparated lymphocytes + PWM. In 2 out of 3 (Rehatuin, Flow and Gibco) instances they exceeded those observed with combinations of B and T cells. Negligible responses were observed in cultures of T cells alone, indicating adequate separation. Coculture with T cells enhanced up to ninefold the B cell response. With the exception of unseparated lymphocytes cultured with the Gibco fetal calf serum, standard errors of the mean for all other groups substantially overlapped, indicating that there was no significant difference in efficacy between the 3 sources of fetal calf serum used - Figure 19.

Conclusion:

This study verified observations made in earlier studies of the effect of T cells and PWM on the B cell response. From the data presented it was concluded that there was no significant difference in efficacy between the 3 sources of fetal calf serum in the vast majority of cultures. For reasons of availability and economics Gibco (Grand Island Biological Company) was chosen as the source of fetal calf serum for all subsequent studies. The higher responses observed for unseparated lymphocytes than B + T cells in the presence of PWM were unaccounted for. Since this trend was reversed in

the absence of PWM it may have represented different dose response characteristics of the two cell populations, or it may have represented a more favourable B;T cell ratio in this subjects unseparated lymphocytes, or a combination of the two.

Study No. 5:

It has been recognized that the subpopulation of T cells known as T suppressor cells are sensitive to irradiation, being functionally destroyed at appropriate dose levels. Another subpopulation, T helper cells are relatively radio-insensitive (Tolerate higher doses before being destroyed). In Study No. 6 functional destruction of T lymphocyte sub-populations by irradiation provided a means by which immunoregulation in rheumatoids and normals could be studied. Reports of the relative radio-sensitivity of these two sub-populations have varied. It was therefore necessary to establish in this laboratory, which dosages of irradiation produced maximal and minimal responses. A single normal (c.c.) and two rheumatoids (B.F., L.H.) were chosen for study.

Original data are displayed in Appendix 3, cultures No. 196-217.

Conditions:

Cells were separated as previously indicated. Aliquots of T cells were irradiated (Gamma Cell, Canada) at varying dosages between 800 and 6,400 rads, and washed as already described prior to coculture with $5x10^4$ B cells (1:1 ratio combination) for a period of 7 days in the presence of 1% PWM (Gibco Lot. No. 15K0201).

Results:

Responses were lower in the 2 rheumatoids than in the normal at all radiation dosages with the exception of $B + T_{6400}$ which fell between the response of the rheumatoids (Table 9). Responses in rheumatoids and in normals were maximal when T cells were irradiated at 3,200 rads prior to coculture. The dose response curves for rheumatoids were significantly flatter than for the normal. i.e. Increasing doses of T cell irradiation up to 3,200 rads produced a greater enhancement in the normal subject than in the rheumatoids (Figure 20). Furthermore, irradiating the T cell population at doses higher than 3,200 rads produced a diminution in the observed responses in both groups. In 2 out of 3 subjects the response at 6,400 rads was less than that at 800 rads.

Conclusion:

Irradiation of T cells prior to coculture with B cells had a significant effect on observed responses, which were maximal at 3,200 rads and minimal at 6,400 rads. Radiation enhancement was greater in the normal than in the rheumatoids. However, L.H. had a significant response to radiation. The maximum level of response in rheumatoids (1332-2600 PFC/10^6 PBL) was less than in the normal (8275 PFC/10^6 PBL). Taken together this evidence suggested that the subpopulation of

TABLE 9. - EFFECT OF T LYMPHOCYTE PRE--IRRADIATION ON IMMUNOGLOBULIN SYNTHESIS IN B & T & PWM CULTURES IN RHEUMATOIDS AND NORMALS.

SUBJECT	RADIATION DOSE	PFC/10^6 PBL $\pm$ SEM
c.c.	800	2921 $\pm$ 118
	1600	4575 $\pm$ 622
	3200	8275 $\pm$ 622
	6400	666 $\pm$ 73
B.F.	800	825 $\pm$ 93
	1600	966 $\pm$ 89
	3200	1332 $\pm$ 108
	6400	90 $\pm$ 27
L.H.	1000	1250 $\pm$ 150
	2000	1900 $\pm$ 0
	3200	3300 $\pm$ 100
	5000	2600 $\pm$ 600
	6400	2300 $\pm$ 700

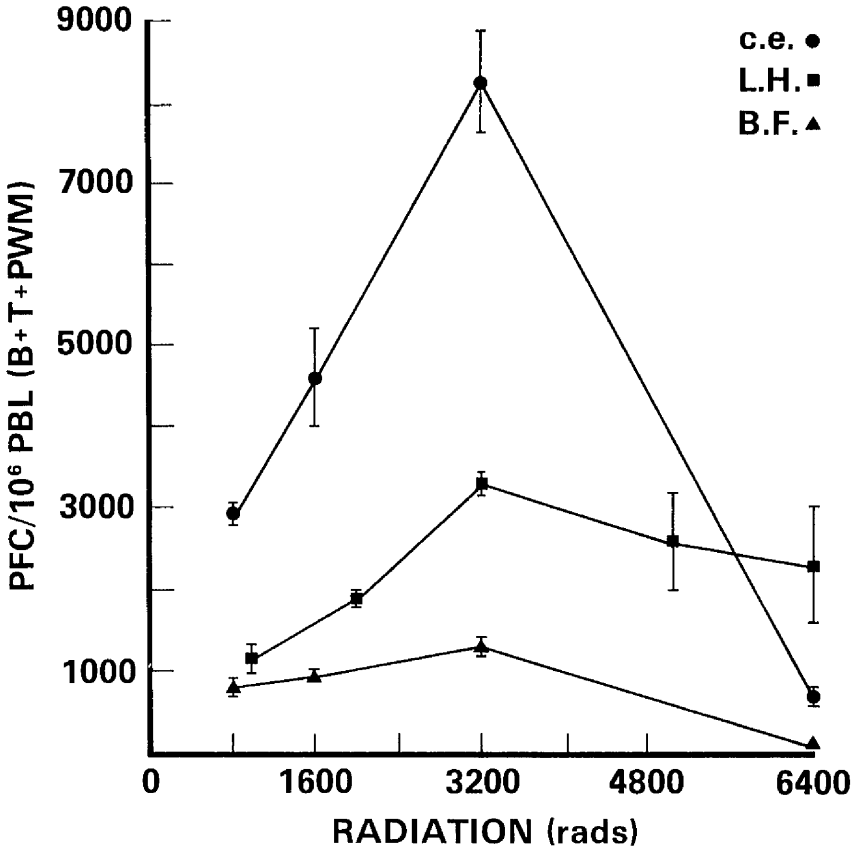


FIGURE 20. - RADIATION DOSE/RESPONSE CURVES IN TWO RHEUMATOIDS (L.H., B.F.) AND A SINGLE NORMAL SUBJECT (c.e.)

T cells known as T suppressor cells were sensitive to irradiation at an optimal dose of 3,200 rads in this laboratory, while higher doses of irradiation (6,400) resulted in a functional destruction of subpopulations containing T suppressor and T helper cells. It was from this study that 3,200 rads and 6,400 rads were chosen as the optimal dosages at which to irradiate T cells in subsequent studies. T cells irradiated at 3,200 rads are hereafter designated as T_{3200} and at 6,400 rads T_{6400} cells.

SUMMARY.

Following a review of available experimental techniques a modified reverse haemolytic plaque forming cell assay was selected to measure Ig synthesis from B lymphocytes. Five studies were conducted to standardize techniques, and in particular to establish optimal culture duration and concentration of pokeweed mitogen, the effect of different sources of foetal calf serum and to define radiation dose response curves for T cells. Cell yield was found adequate and provided sufficient numbers of cells for replication of the necessary cell culture combinations. Furthermore, cell viability was well maintained, being greater than 95% at the time of culture and 55-80% at the time of plaquing. Consistent with the observations of others, the PFC response was maximal after 7 days of culture. Dose response curves for pokeweed mitogen indicated a need to operate on the plateau of the curve for maximal responses and justified the selection of 1% as the standard concentration.

However, responses from two separate lots of pokeweed mitogen differed, suggesting that the same lot number should be used for all experiments or if it were changed, then caution should be exercised in combining results from studies using these different lot numbers. Since Gibco FCS was readily available and compared favourably with other sources of foetal calf serum it was selected for all subsequent studies. Consistent with the literature, suppressor T cells were found to be more radiosensitive (3200 rads), than helper T cells (6,400 rads). When

T cells were pre-irradiated at 3200 rads prior to coculture with B cells, responses from normal subjects and response differences between rheumatoids and normal subjects were maximal. Thus 3200 rads and 6400 rads were selected as the standard dosages with which to manipulate the helper suppressor balance. While certain trends were evident in the early experiments, the small numbers of subjects studied prohibited conclusions being drawn. Therefore study 6 was designed, incorporating the experience gained in the first 5 experiments and involving large number of rheumatoid and normal subjects.

CHAPTER 4

EVALUATION OF THE CONTROL OF POLYCLONAL IMMUNOGLOBULIN SYNTHESIS IN RHEUMATOID ARTHRITIS.

(STUDY 6)

- INTRODUCTION
- SUBJECTS AND METHODS
- RESULTS
- DISCUSSION
- SUMMARY

With signs and symptoms processed in computers
With patients' histories on punched-cards coded,
Old teaching ways ere long will be outmoded.
For philosophic profs, loquacious tutors,
Their classes' cool derision won't be spared
If they dilate on clinical impression
And quote no coefficients of regression,
Significance with t-test or chi-squared
Or standard deviations (i.e. sigma)
Mandatory now in scientific thinking -
Clinicians' aphorisms not hoodwinking
The unbelieving young; 'folk-lore' their stigma,
Technocracy ascends: it is our fate
Illiterate to be, but numberate.

Anon.

INTRODUCTION:

While a large number of studies (Chapter 2) have defined immune abnormalities in rheumatoid arthritis, relatively few have specifically examined the control of the immune response; an enterprise which may provide explanation for some observations. Research conducted on several non-rheumatoid "autoimmune" diseases indicates that disturbances of immunoregulation are not disease specific, but may be found in more than one disease state. Thus, immunoregulatory abnormalities have been noted in a number of such disorders[248] including systemic lupus erythematosus (SLE)[249], juvenile rheumatoid arthritis (JRA)[250], sarcoidosis[251], mixed connective tissue disease (MCTD)[252] and Sjogren's syndrome[253]. In human[249, 254, 255, 256] and murine[257] lupus syndromes, alterations in T cell function for help and more especially suppression have been observed. Hypofunction in suppressor T cells has been noted in some polyclonal gammopathies[258], while hyperfunction has been described in common variable hypogammaglobulinemia[259]. Although antisuppressor cell antibodies have been demonstrated in the sera of patients with active SLE[255, 256] and JRA[250] the mechanisms responsible for suppressor T cell dysfunction remain unknown.

Recently two groups examining immunoregulation in rheumatoid peripheral blood, utilising different techniques have attributed deviations from the normal in their experimental endpoints to suppressor T cell dysfunction[260, 261, 262]. Abdou et al[261] studied

31 patients with varying severities, activities and durations of definite or classical rheumatoid arthritis, 11 patients with osteoarthritis and 34 normal healthy controls. All patients with rheumatoid and osteoarthritis were receiving some form of anti-inflammatory therapy, commonly aspirin. Lymphocytes were activated with Conconavilin A. These authors reported that suppressor T cell hypofunction was found in active early rheumatoid arthritis (disease less than 3 months) but not in active late (greater than 6 months) or inactive R.A. When these active early rheumatoids were restudied 11-23 months later, suppressor cell function had normalised, in spite of persistence of disease activity. Furthermore, antisuppressor cell antibody of IgG class was detected in plasma from active early R.A. patients but not in the plasma from other R.A. groups or non-R.A. controls. The B cell targets for Conconavilin A activated suppressor cells were functionally normal in early active R.A., confirming the initial impression that the defect lay at the level of the T cell and not in the capacity of B cells to respond to suppressor signals. Finally, responses from osteoarthritic and normal subjects were similar, indicating that the observed responses were not the consequence of a non-specific drug effect. This finding contrasted with that of other workers[263, 264, 265] who have reported an effect of non-steroidal anti-inflammatory therapy on other in vitro experimental endpoints (tritiated thymidine incorporation).

Abdou et al concluded that suppressor cell hypofunction may contribute to the initiation but not the persistence of rheumatoid arthritis.

Keystone et al[262] studied 20 patients with rheumatoid arthritis and 16 age and sex matched healthy controls. Suppressor cell activity was generated in vitro by priming peripheral blood mononuclear cells with high dose antigen (ovalbumin) and measuring the subsequent response after culture in an ovalbumin-specific PFC assay. The authors observed, that while the mean PFC response in rheumatoids and normals were similar, that suppressor cell activity in rheumatoids was less ($46.4 \pm 4.2\%$) than in controls ($64.6 \pm 2.7\%$). No correlation was noted between suppressor cell activity and disease activity or drug therapy. A pure hypofunction of suppressor activity would be expected to enhance the PFC response. The authors resolved this apparent paradox by suggesting that antibody synthesis was dependent upon complex interactions of accessory, helper, suppressor and B cells, and that impaired function of cells which augmented the PFC response may have offset the suppressor defect.

There are no previous reports in the literature of the reverse haemolytic plaque forming cell assay and radiation enhancement techniques being used systematically to evaluate immunoregulation in rheumatoid arthritis. In the study to be described polyclonal immunoglobulin synthesis by rheumatoid

B cells was compared with that from normal B cells. In order to examine the function of lymphocyte subsets, autologous cultures of B cells and irradiated (T_{3200} of T_{6400}) and non-irradiated T cells were established in 1:1 ratio combinations. Cultures of B cells alone and T cells alone (non-irradiated, T_{3200} and T_{6400}) were included to allow further comparisons to be made and basal levels of immunoglobulin synthesis to be defined in this system. Finally, unseparated lymphocytes were cultured to compare responses of immuno-competent cells when present in proportions existing in the peripheral blood, as opposed to responses seen in the 1:1 ratio (B:T) combinations created in the laboratory. Patients of varying age, sex, rheumatoid factor status, disease activity, disease duration and therapeutic regimen were selected in order to examine a cross section of the rheumatoid population, and provide opportunity to recognize any factors which might determine a response deviating from the normal. Simultaneous with this experiment, data were gathered on Tγ cells. Since these data have particular significance and were subsequently added to, they will not be dealt with in this section but instead reported in Chapter 5. An outline description of the methods used is repeated, in order to clearly define the incorporation of results from studies 1-5 in the final design of study 6.

SUBJECTS AND METHODS.

Thirteen patients who fulfilled A.R.A. criteria for classic or definite rheumatoid arthritis (MW, JF, BF, BL, HM, EB, SS, LH, HB, DB, CM, SW, DM) and fourteen normal subjects (nb, ke, lg, ds, ec, db, cm, ww, jh, jd, mm, ej, bw, wc) were selected for study. (Appendices 1 and 2). Mean disease duration was 9.3 years (range 4 months to 23 years). Eleven patients were female and the same number seropositive. Twelve patients were receiving nonsteroidal anti-inflammatory drugs at the time of study, four were additionally receiving Prednisone and/or gold or Penicillamine. One female rheumatoid was taking only acetaminophen and was recorded for the purposes of analysis as "no treatment". The A,R.A. functional class of patients was assessed by a physiotherapist and ESR measured by the Westergren method. Rheumatoid factor titres were determined by the latex method.

None of the fourteen normal subjects was known to be taking medication at the time of the study or be suffering from a systemic disease, or microbial infection. Fifty per cent of control subjects were female.

Mononuclear cells were separated from 30 mls of fresh heparanized blood by Ficoll-Hypaque (Ficoll 400, Pharmacia Fine Chemicals AB Uppsala, Sweden) (Hypaque, Winthrop Laboratories, Aurora, Ontario) (SG 1.079-1.084) centrifugation (800g for 15

minutes at 4°C), and washed three times inRPMI 1640 pH 7.4 (Grand Island Biological Co., Grand Island, New York) with resuspension following centrifugation (300g for 8 minutes at 4°C). An aliquot of unseparated mononuclear cells was retained and from the remaining cell suspension adherent (B) and non-adherent (T) cells were separated using a modification of the nylon wool technique described by Werner et al. Columns were prepared as previously described, and rinsed through with 10mls RPMI 1640 and then 3mls of 5% v/v fetal calf serum (Grand Island Biological Co., New York), following which they were placed in a 37°C incubator for 30 minutes. Peripheral blood mononuclear cells in 0.5 mls of 5% fetal calf serum in RPMI 1640 at 37°C were loaded on to each column and incubated for half an hour. Nonadherent cells were removed by running 10mls of prewarmed 37°C 5% fetal calf serum in RPMI 1640 through the column. Adherent cells were removed by rinsing the column with 3x1.5mls of warmed 5% fetal calf serum in RPMI 1640 while gently massaging the tube. The cells were thereafter washed twice in RPMI 1640 and resuspended, following centrifugation at 300g for 8 minutes at 4°C. 98% of nonadherent cells, rosetted with sheep red blood cells, whereas less than 1% of adherent cells (70-80% sIg positive) rosetted, indicating effective separation of B and T lymphocytes. The yield of B and T lymphocytes in R.A. and normals was similar (approximately 85%).

Aliquots of nylon passed (T) cells were either non-irradiated or irradiated at one of two dosages; 3200 rads (to eliminate T suppressor cells) and 6400 rads (to eliminate T helper and T suppressor cells). The rational for choosing these dosages was outlined in the previous chapter. $5x10^4$ mononuclear cells were cultured in replicate either as T cells alone (either non-irradiated or irradiated at 3,200 or 6,400 rads) or as B cells alone, or in a 1:1 ratio.combination of T cells (which were either non-irradiated or irradiated at 3,200 or 6,400 rads) with B cells. Additional cultures contained 10^5 unseparated mononuclear cells. Cells were incubated for 7 days (peak period of response) at 37°C in 1 ml of RPMI 1640 (containing L glutamine) supplemented with 10% heat inactivated sheep red blood cell absorbed fetal calf serum (Grand Island Biological Company, Grand Island, New York) and gentamicin 50 mcg/ml (Schering Corporation, Kenilworth, New Jersey) both with and without PWM at an optimal concentration of 1% v/v (PWM - Grand Island Biological Company, New York, Lot #15K0201). Cultures were contained within 12 x 75 mm plastic tubes (Falcon Plastic, Cockeysville, Maryland) and maintained in a humidified environment containing 5% CO_2. Following incubation the cells were washed three times in RPMI 1640, resuspended in 0.5 mls of BSS pH 7.4 following centrifugation (300g for 8 minutes at 4°C) and the number of viable lymphocytes counted by a trypan blue staining method. Immunoglobulin synthesis by B cells, unseparated mononuclear cells and various combinations of B and T cells was measured using a

reverse haemolytic plaque forming cell assay as described in the preceding chapter. However, in contrast to earlier studies, a constant 100 µl volume of lymphocyte suspension was used, being mixed with agarose and coated SRBC (as previously described) prior to introduction into the assay. Plaque forming cells were counted and expressed per 10^6 viable (by trypan blue staining) peripheral blood lymphocytes. Data were analysed by an unpaired t-test and p values less than 0.05 considered significant.

RESULTS.

Original data from replicate determinations are tabulated in Appendix 3 (cultures No. 218-767), while individual means for rheumatoids are tabulated in Table 10 and for normals in Table 11. Results are expressed as plaque forming cells per 10^6 viable peripheral blood lymphocytes (mononuclear cells) $\pm$ SEM (the standard error of the mean). Missing data points are entered as (-) while a result of zero is entered as (0).

A range of responses was described for each culture combination in both rheumatoids and normals, with and without pokeweed mitogen. PFC responses to unseparated lymphocytes in the presence of PWM ranged 500 - 3,522 (+ PWM), 250 - 1289 (- PWM) in R.A. and 820 - 6746 (+ PWM), 600 - 3750 (-PWM) in normals. Responses for B cells only ranged 100 - 1450 (+ PWM), 0 - 1000 (- PWM) in rheumatoids and 59 - 775 (+ PWM), 50 - 450 (- PWM) in normals. Responses for B + T (nonirradiated) ranged 850 - 6600 (+ PWM), 250 - 1650 (- PWM) in R.A. and 1200 - 5625 (+ PWM),

TABLE 10 - PFC RESPONSE AND RADIATION ENHANCEMENT IN RHEUMATOIDS (INDIVIDUAL MEANS ± SEM)

CELLS	UNSEP		B		B & T		B & T3200		B & T6400		T		T3200		T6400	
PWM	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
MW	3522	650	1450	500	6600	800	5000	1500	2750	500	33	0	50	0	0	0
JF	500	250	200	150	850	500	600	400	200	200	0	0	0	0	0	0
BF	1550	1150	300	100	1450	700	1700	600	850	834	0	0	0	0	0	0
EL	1000	550	200	150	1200	500	1500	400	630	500	0	0	0	0	0	0
HM	2579	1289	-	72	2247	268	2210	997	1679	661	0	0	0	0	0	0
EB	1463	364	1000	357	2829	1650	2697	464	643	321	0	0	0	0	0	0
SS	1232	389	570	1000	1232	848	1247	710	456	429	0	0	0	0	0	0
LH	-	-	754	150	1270	250	3300	200	2300	200	50	-	-	-	-	-
HB	-	-	100	0	2160	262	1833	257	-	-	0	0	-	-	-	-
DB	-	-	750	150	2460	1050	3037	800	3350	1550	0	0	-	-	-	-
CM	-	-	350	200	1150	325	-	-	-	-	0	0	-	-	-	-
SW	-	-	238	143	4090	572	3718	572	1022	429	-	-	-	-	-	-
DM	-	-	1000	143	3159	1367	3785	1500	1071	786	0	0	-	-	-	-
MEAN	1692	663	576	260	2361	699	2552	700	1361	555	7	0	7	0	0	0
SEM	±388	±153	±121	±76	±440	±122	±366	±126	±309	±133	±5	±0	±7	±0	±0	±0

TABLE 11 - PFC RESPONSE AND RADIATION ENHANCEMENT IN NORMALS (INDIVIDUAL MEANS ± SEM)

CELLS	UNSEP		B		B & T		B & T3200		B & T6400		T		T3200		T6400	
PWM	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
nb	6500	3750	300	250	5625	3300	10600	7050	3650	950	100	100	0	200	200	0
ke	6746	1175	650	450	1950	550	16400	6050	1700	1800	0	200	100	0	0	0
lg	1680	975	775	300	1330	759	9191	725	1450	600	141	95	100	125	100	75
ds	750	700	350	1200	800	8600		3800	1350	850	0	0	0	0	0	0
ec	1550	600	550	300	1250	550	3700	1000	550	300	-	-	-	-	-	-
db	2116	1450	350	50	2500	1000	7900	1450	2200	900	0	0	0	0	0	0
em	820	705	455	175	1338	373	3605	1427	614	231	0	0	0	0	0	0
ww	-	-	431	-	1600	100	4525	-	1475	100	200	50	0	0	0	0
jh	3844	742	643	100	2491	750	8750	2600	3000	330	-	-	-	-	-	-
jd	-	-	200	50	2175	264	4190	600	1040	132	0	0	-	-	-	-
mm	-	-	213	-	3428	1571	8228	2857	1047	500	0	0	-	-	-	-
ej	-	-	59	-	2244	257	2208	429	-	-	0	0	-	-	-	-
bw	-	-	107	-	1676	-	2631	629	-	-	0	0	-	-	-	-
wc	1550	600	550	300	1250	550	3700	1000	550	300	0	0	0	0	0	0
MEAN	2923	1194	427	233	2147	833	6731	2278	1569	583	37	37	25	41	38	10
SEM	±752	±333	±61	±43	±318	±231	±1055	±598	±280	±140	±20	±20	±16	±28	±26	±9

100 - 3300 (-PWM). Responses for B + T_{3200} ranged 600 - 5000 (+ PWM), 200 -1500 (-PWM) in rheumatoids. and 2208 - 16400 (+PWM), 429 - 7050 (- PWM) in normals. Finally, responses for B + T_{6400} ranged 200 - 3350 (+ PWM), 200 - 1550 (-PWM) in R.A. and 550 - 3650 (+ PWM) 100 - 1800 (- PWM). Responses from T cells alone were usually 0,rarely above 150 and never above 250. Thus it was evident that for each culture combination there was some degree of overlap. However, a previously noted trend emerged i.e. responses from T cells were negligible, responses from B cells alone were in the low range, responses from B + T and unseparated lymphocyte cultures were in the mid range, and B + T_{3200} + PWM cultures had a range, the upper extent of which in normals exceeded that of all other rheumatoid and control cultures. In order to compare results from rheumatoids and normals group means $\pm$ the standard error of the mean (SEM) were calculated (Table 12), and enhancement factors derived by dividing the PFC response for B + T_{3200} + PWM by the PFC response for B + T + PWM for each individual, i.e.

$$\text{Enhancement factor} = \frac{B + T_{3200} + PWM}{B + T + PWM}$$

Mean responses from T cells cultured alone were negligible in both rheumatoids (< 50) and normals (< 50) indicating effective separation of B cells from T cells. These minimal responses were unaffected by T cell radiation at a dose (6400 rads), which would

TABLE 12 - PFC RESPONSE AND RADIATION ENHANCEMENT IN RHEUMATOIDS AND NORMALS (GROUP MEANS ± SEM)

CELL COMBINATION	PWM	PFC/10^6 PBL ± SEM		
		NORMAL (n=14)	R.A. (n=13)	p
UNSEP	+	2923 ± 752	1692 ± 388	NS
	-	1194 ± 333	663 ± 153	NS
B	+	427 ± 61	576 ± 121	NS
	-	233 ± 43	260 ± 76	NS
B + T	+	2147 ± 318	2361 ± 440	NS
	-	833 ± 231	699 ± 122	NS
R+T3200	+	6731 ± 1055	2552 ± 366	<0.005
	-	2278 ± 598	700 ± 126	<0.025
R+T6400	+	1569 ± 280	1361 ± 309	NS
	-	583 ± 140	550 ± 133	NS
T	+	37 ± 20	7 ± 5	-
	-	37 ± 20	0 ± 0	-
T3200	+	25 ± 16	7 ± 7	-
	-	41 ± 28	0 ± 0	-
T6400	+	38 ± 26	0 ± 0	-
	-	10 ± 9	0 ± 0	-

functionally destroy any contaminating B cells together with T suppressor and radiosensitive T helper cells, and can therefore be taken as the background response of the assay. Moreover, since plaque numbers were multiplied to derive responses per 10^6 PBL they actually represented the presence of very few plaques.

Responses from B cells cultured alone were similar in rheumatoids (576 $\pm$ 121) and normals (427 $\pm$ 61) in the presence of PWM and were comparable in its absence (260 $\pm$ 276 and 233 $\pm$ 43 respectively), although at lower response levels. When non-irradiated T cells were cocultured with B cells there was significant (approximately four-fold) enhancement of immunoglobulin synthesis (Figure 21) by both rheumatoids (2361 $\pm$ 440) and normals (2147 $\pm$ 318) in the presence of PWM as well as in its absence (699 $\pm$ 122 and 833 $\pm$ 231 respectively). Again there was no statistically significant difference between the two groups.

However, when T cells were irradiated at 3200 rads prior to coculture, further marked enhancement was noted (over B + non-irradiated T response levels) in immunoglobulin synthesis in normals (6731 $\pm$ 1055), whereas in rheumatoids enhancement was absent or minimal in ninety per cent of patients studied (2552 $\pm$ 366). Even in the absence of PWM, enhancement was noted in normals (2278 $\pm$ 598) but not in rheumatoids (700 $\pm$ 126). These differences in radiation enhancement were statistically significant both with,

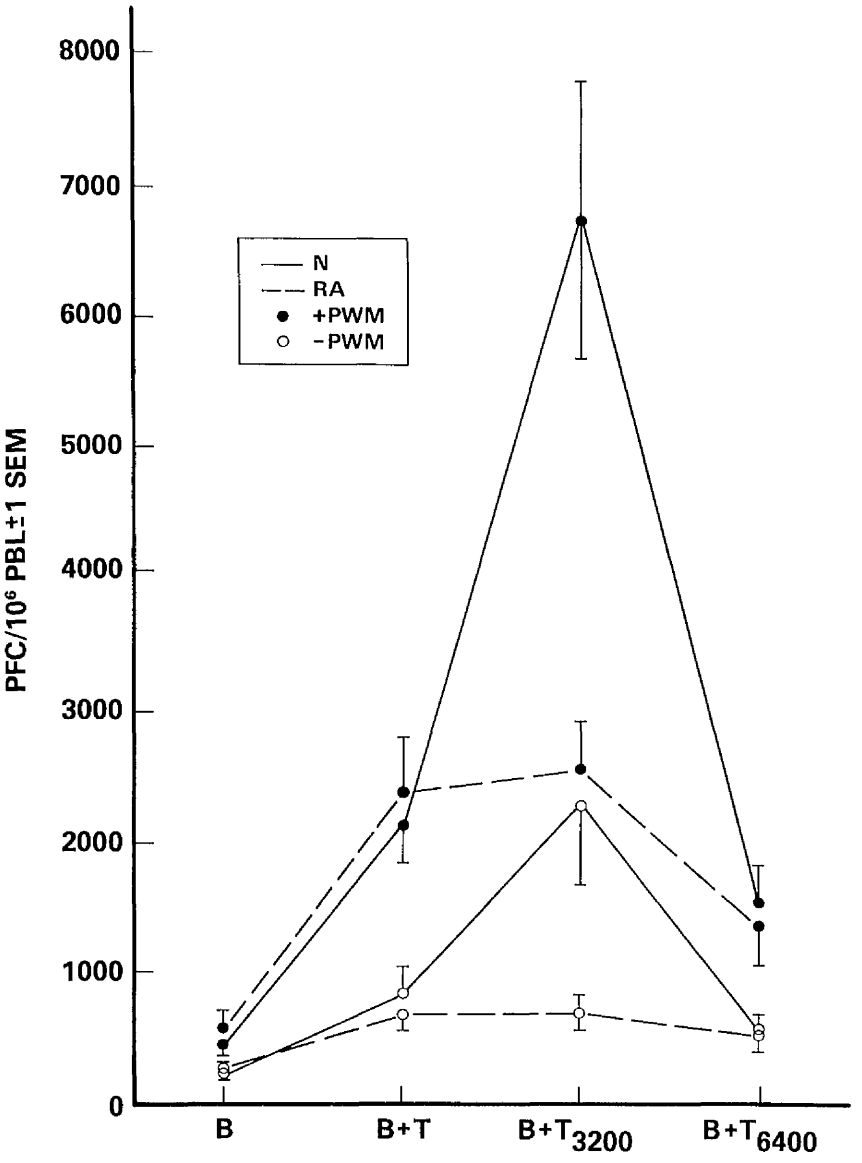


FIGURE 21.- Immunoglobulin synthesis (PFC, mean ± SEM) in lymphocyte cultures from rheumatoid and normal subjects with and without pokeweed mitogen.

($p < 0.005$), and without, ($p < 0.026$) PWM. Enhancement factors in rheumatoids varied from 0.71 - 2.6 (mean 1.1) and in normals from 0.98 - 8.4 (mean 3.53). (Table 13). While ranges in the two groups overlapped it was evident that the majority of normal subjects had an enhancement factor greater than 2, a value which was achieved by only one rheumatoid. In the majority of rheumatoids enhancement was less than 1.5 and often less than 1. (Using the enhancement factor calculation, a value of < 1 represented no enhancement).

When T cells were irradiated at 6400 rads prior to coculture responses were less than with cultures of B + non-irradiated T cells but greater than responses from cultures of B cells alone. There was no significant difference in responses between rheumatoids and normals both with (1361 ± 309 and 1569 ± 280 respectively), and without, (555 ± 133 and 583 ± 140 respectively) PWM.

Responses from unseparated lymphocytes were similar in both patients (1692 ± 388) and normals (2923 ± 752). Although unstimulated responses were lower in rheumatoids (663 ± 153), there was no statistically significant difference between patients and controls (1194 ± 333).

Thus statistically significant differences in immunoglobulin synthesis between the two groups was shown only when B cells were cocultured with T cells irradiated at 3200 rads. The doses of

TABLE 13. - ENHANCEMENT FACTORS FOR RHEUMATOID AND NORMAL SUBJECTS. (RANKED)

Rheumatoid Arthritis		Normal	
LH	2.6	ke	8.4
BL	1.3	ds	7.2
DB	1.2	lg	6.9
DM	1.2	jh	3.5
BF	1.2	db	3.2
SS	1.01	ec	3.0
HM	0.98	wc	3.0
EB	0.95	ww	2.8
SW	0.91	cm	2.7
HB	0.85	mm	2.4
MW	0.76	jd	1.9
JF	0.71	nb	1.9
		bw	1.6
		ej	0.98
Mean = 1.1		Mean = 3.53	

irradiation used produced no observable alteration in cell viability, which was better than 98% preculture and 70% post-culture for combinations of all cell types (irradiated and non-irradiated).

While it was evident that a range of responses existed for both normals and rheumatoids, it was clear that radiation enhancement was defective in the rheumatoid population (Figure 22). The relationship between radiation enhancement and several variables (drug therapy, disease duration, disease activity as reflected by ESR, sex, age and rheumatoid factor status) was examined.

A review of drug therapy (Table 14) identified those rheumatoids taking only non-steroidal anti-inflammatory agents (8), the single subject taking acetaminophen (1) and those taking a non-steroidal anti-inflammatory agent as well as prednisone or a remittive agent (4). Subsequent analysis by drug therapy (Figure 23), failed to review any consistent relationship between any particular drug group and the degree of radiation enhancement. The maximum enhancement factor of 2.6 was observed in a patient (LH) on a non-steroidal anti-inflammatory drug, while a complete failure of enhancement was recorded in a patient (EB) receiving no anti-inflammatory medication. None of the controls used in the present study were on medication at the time of study, however, evidence will be cited later to indicate that normal subjects on non-steroidal anti-inflammatory drugs show normal enhancement.

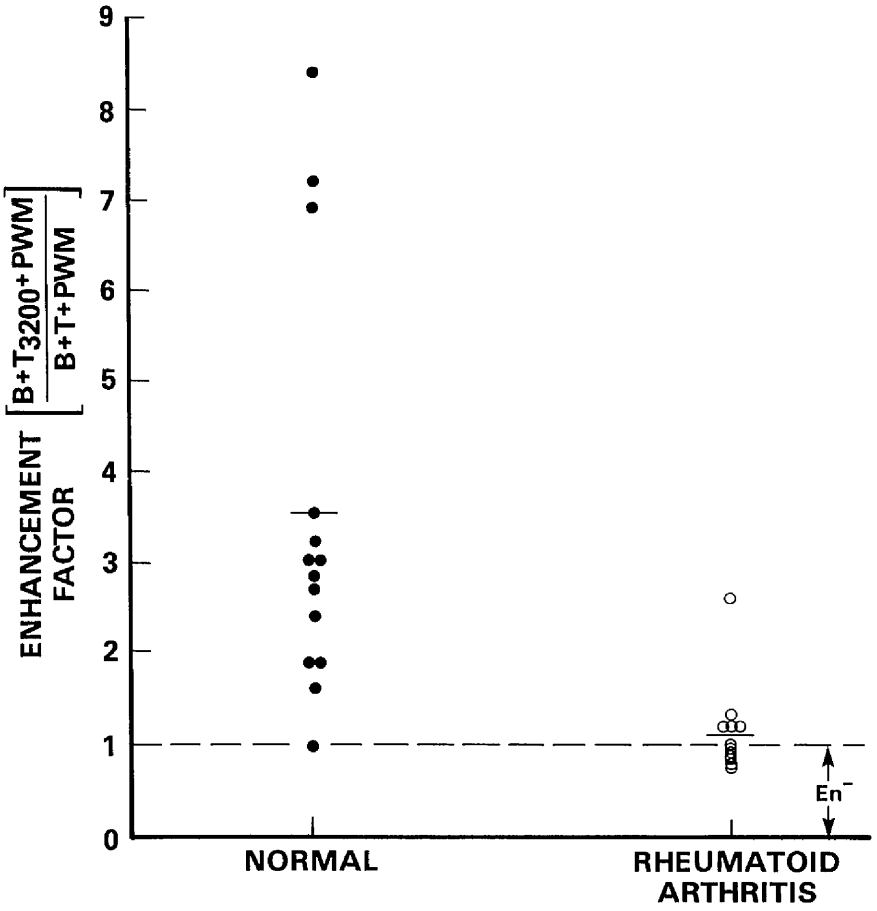


FIGURE 22. - Comparison of enhancement factors in normals and rheumatoid arthritis. (En- = Enhancement Failure)

TABLE 14 - DEMOGRAPHIC PROFILES AND THERAPEUTIC REGIMENS OF RHEUMATOIDS.

PATIENT	TREATMENT REGIMEN				AGE	SEX	DISEASE DURATION (YRS)	ESR	RF
	NSAID	PRED	CHLORO-QUIN.	Au/D-P					
MW	+	-	-	+	66	F	4	60	+
JF	+	-	-	-	79	F	0.5	56	-
BF	+	-	-	-	64	M	16	24	+
BL	+	+	-	-	59	F	17	66	+
HM	+	-	-	-	56	F	10	50	+
EB	-	-	-	-	39	F	11	14	-
SS	+	-	-	-	86	F	0.4	50	+
LH	+	-	-	-	31	F	4	13	+
HB	+	+	-	+	54	F	23	39	+
DB	+	-	-	-	56	F	0.8	46	+
CM	+	+	-	+	62	F	27	34	+
SW	+	-	-	-	59	M	0.3	48	+
DM	+	-	-	-	56	M	8	42	+

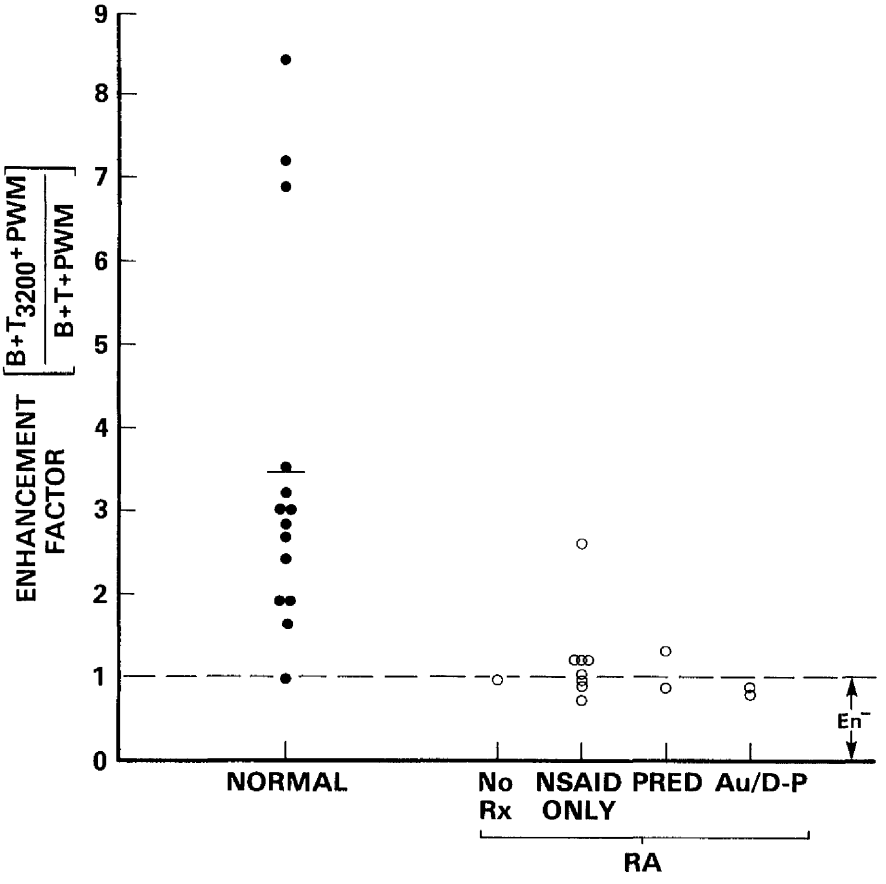


FIGURE 23.- Correlation of enhancement factor with drug regimen in rheumatoid arthritis. (En- = Enhancement Failure)

When disease duration was considered (Figure 24), no association was found with radiation enhancement. It was noted that in two patients who had had the disease for four years, one had an enhancement factor of 2.6, whilst the other was 0. It was also noted that a patient who had had the disease for sixteen years, had a minimal enhancement of 1.2, while two patients who had had the disease for only a few months showed no enhancement.

The erythrocyte sedimentation rate (ESR) determined by the Westergren method was used as one measure of disease activity (Figure 25). No association was shown between ESR and enhancement factor; mild, moderate and marked elevations of the ESR all being associated with no or negligible enhancement. Of two patients with an ESR less than 20 mms in the first hour, one showed an enhancement factor of 2.6 while the other failed to enhance.

Finally, no association was shown between enhancement factor and sex, age or rheumatoid factor status (Figure 26). Males and females, and patients less than 60 and greater than 60 years of age showed equivalent ranges of enhancement factors. The highest enhancement factor was in a young seropositive female. Only two seronegative patients were studied, both of whom failed to enhance, However, because of the small numbers, no inference can be drawn from this finding.

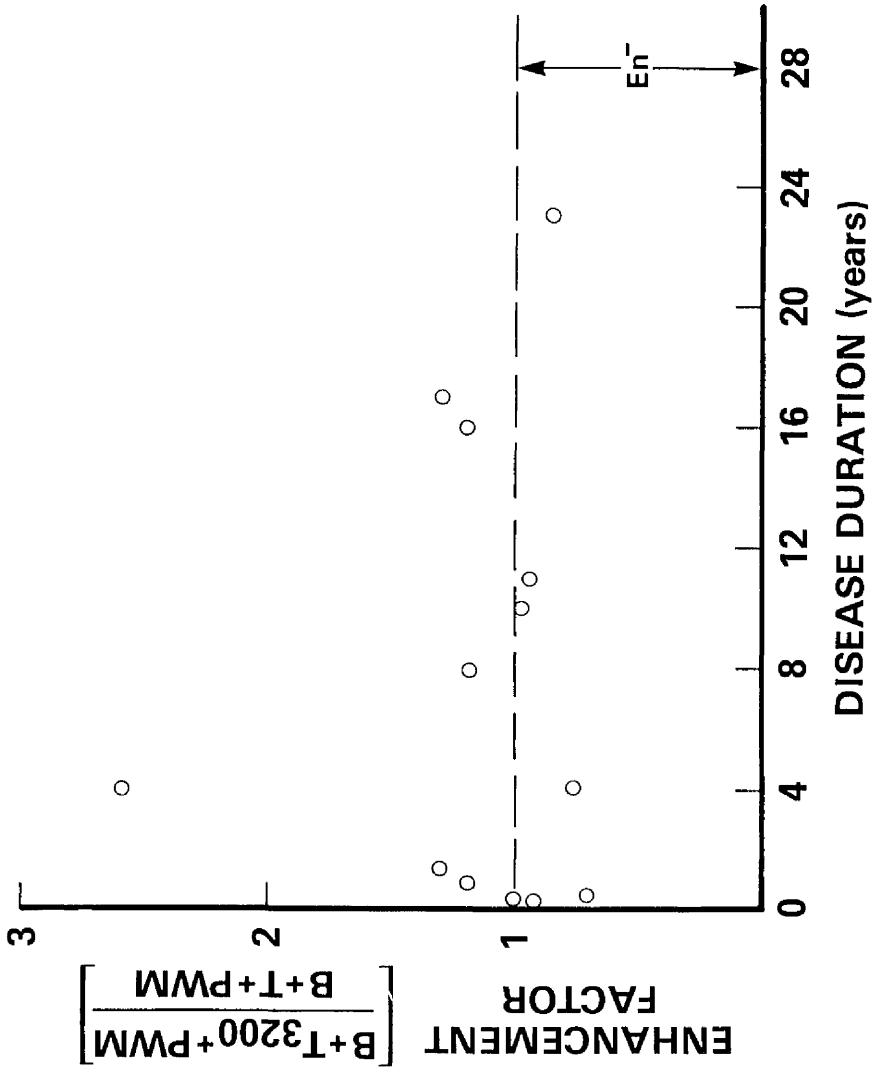


FIGURE 24. - Correlation of enhancement factor with disease duration (years) in rheumatoid arthritis. (En- = Enhancement Failure)

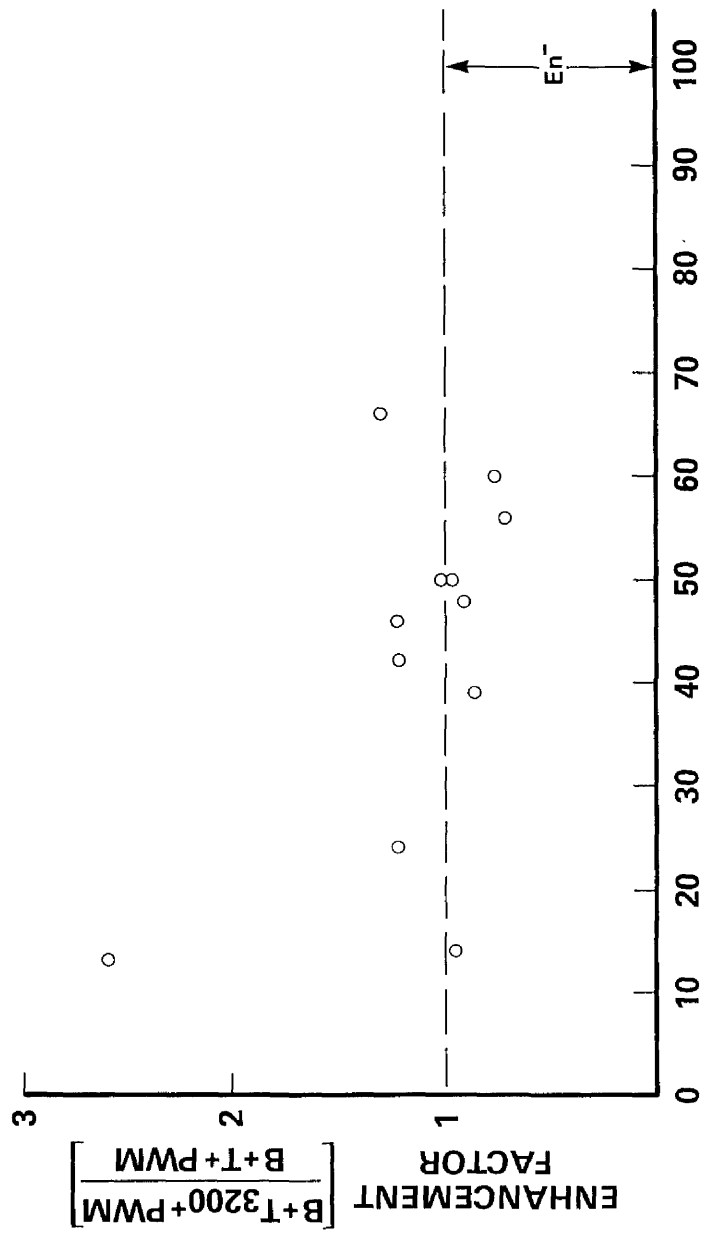


FIGURE 25. - Correlation of enhancement factor with erythrocyte sedimentation rate (ESR) by the Westergren technique (mm/1st hr) in rheumatoid arthritis.
(En- = Enhancement Failure)

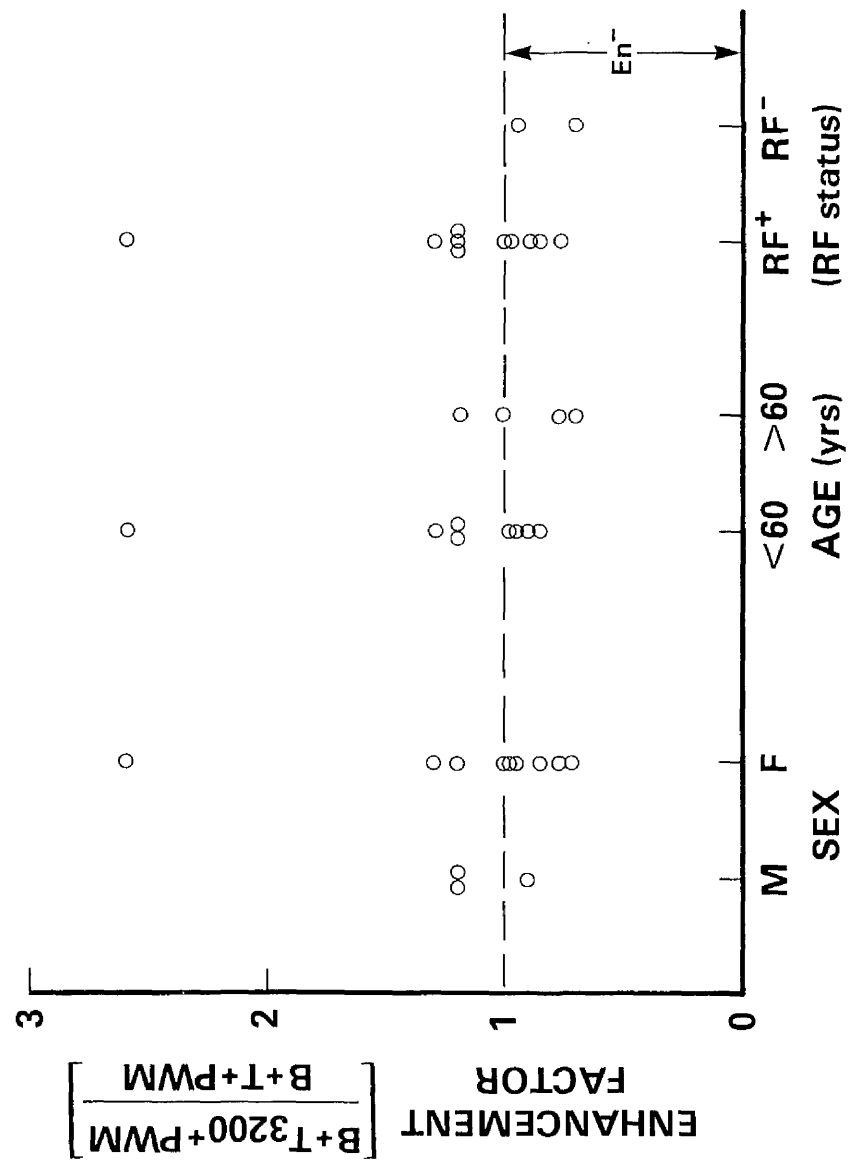


FIGURE 26. - Correlation of enhancement factor with sex, age and IgM rheumatoid factor status in rheumatoid arthritis. (En- = Enhancement Failure)

DISCUSSION.

The effects of pokeweed mitogen, T cell pre-irradiation and coculture of B cells with T cells on immunoglobulin synthesis by B cells in this study were consistent with those observed in studies 1-5. Low responses from B cells alone and negligible responses from T cells alone, indicated effective cell separation in this T dependent B cell response. Equivalent degrees of enhancement in immunoglobulin synthesis observed in rheumatoids and normals when B cells were cocultured with T cells, indicated that these cells were capable of responding to T helper signals.

Significant (greater than x 2) radiation enhancement was noted in the majority of normals but in only one rheumatoid. It was reasonable from the evidence cited previously to suppose that in normal culture, optimal dose radiation destroyed a functional subset of lymphocytes whose net effect was that of suppression and that this manoeuvre tipped the regulatory balance in favour of help. This effect was not observed in rheumatoids suggesting several possibilities.

1. Decrease in the number of function of suppressor T cells.
2. Decrease in the number or function of helper T cells.
3. Decrease in the number and/or function of helper and suppressor T cells.
4. B cells were restricted in their capacity to respond to helper signals.

5. Rheumatoid monocytes were reduced in numbers or functions.

6. There was a failure in the release or action of important soluble helper factors.

7. The observed response was a laboratory artefact.

While hypofunctional suppressor cells have been reported by other authors, this defect alone would not adequately account for the results of this study. Although radiation enhancement may fail as a consequence of irradiating already hypofunctioning suppressor cells (and thus producing no alteration in the help/suppression balance), the observation that responses from rheumatoid cocultures of B and non-irradiated T cells were the same, rather than greater than those from normals, suggested alternative explanation.

If help alone or help and suppression both were restricted (but not absent) either because of a reduction in numbers or the function of cells, then coculture of nonirradiated T cells with B cells would be expected to enhance the B cell only response, while coculture with T3200 cells might not have any significant additional effect. This response would be consistent with the observed results. However, if rheumatoid B cells were hyporesponsive to helper signals, or if R.A. monocytes were restricted in their activity, or if R.A. T cells failed to release potent soluble helper factors in the normal amount necessary for effective

cell-cell communication then the effects would likely be seen as a reduction but not absolute absence of helper activity.

A final consideration was that the observed responses might be a function of the method used, rather than due to immunoregulatory differences between rheumatoids and normals. Several authors[266, 267, 268], using rat lymphoid tissue and human peripheral blood, have recognized that some cells having suppressor activity adhere to nylon wool columns and that while some of these cells might be monocytes, others are likely suppressor T cells. Saxon and Stevens[269] have suggested that the suppressor function of nylon wool passed cells is not deficient unless the suppressor cells are already activated. It therefore appears possible, that the non-adherent (nylon wool passed) fraction is depleted of B cells, monocytes and a proportion of T cells. Thus a difference in the adherence properties of rheumatoid and normal T lymphocytes might result in in vitro differences not truly representative of the in vivo situation. However, since responses from B cells alone and B + non-irradiated T combinations were similar in this study in rheumatoids and normals, it seemed improbable that differences were artefactual. To further evaluate the composition of the T cell preparation being used, monocytes, Tγ cells, OKT4+ and OKT5+ cells were enumerated in subsequent experiments.

A second consideration concerned observed differences in radioresistance with activation. It has been observed in mice[270, 271], that the radioresistance of certain cells appears to change with activation. Thus, when conconavilin A generated suppressor cells are pre-irradiated (prior to activation) at optimal dosages, the loss of suppression is much greater than with the same dose of irradiation given 48 hours after activation. This suggests that radioresistance is increased in those suppressor cells inducable by Conconavilin A. Since T lymphocytes from rheumatoids and normals was simultaneously irradiated at time 0 in study 6 and the dose response curves of rheumatoids and normals were known (study 5), there was no opportunity for any temporal effect on radiosensitivity to confound the results of this study.

Pre-irradiation of T cells at 6400 rads, resulted in a decline of immunoglobulin synthesis from the levels achieved with B + T3200 cell combination to between those achieved from B cells and B + non-irradiated T cells. Since the response did not return to baseline, the presence of either a radioresistant population of T helper cells or of a minimal helper effect of irradiated T cells was suggested. Immunoglobulin synthesis from unseparated lymphocytes was similar in rheumatoids and normals, suggesting that optimal dose radiation was necessary to uncover differences, which could be detected with this assay.

When examined within group, there was no correlation between the degree of radiation and enhancement and demographic characteristics such as age and sex. Although all but one patient was on anti-inflammatory therapy, there was no association between the type of drug therapy and hyporesponsiveness, suggesting that it was not purely a drug effect. Furthermore, Keystone et al in their essay noted no association between drug therapy and hypofunctional suppressor activity. Normal responses from a rheumatoid on a non-steroidal anti-inflammatory drug and in Chapter 11 a normal subject on Ibuprofen support the notion that enhancement failure cannot be attributed to an effect of therapy.

The lack of correlation with disease duration contrasted with the time - dependent observations of Abdou et al, in that in the London rheumatoids, hyporesponsiveness was seen at all levels of disease duration. Although serial studies were not conducted on the same patients, and while the erythrocyte sedimentation rate is not a particularly accurate measure of disease activity, the failure to show any correlation between radiation enhancement and ESR may indicate that the defect is primary or if secondary, that it does mirror disease activity. Examination of the relationship between enhancement and IgM rheumatoid factor status did not indicate that enhancement

failure was more or less common in seropositive patients.

Thus, it was evident from this study that consistent differences in radiation enhancement existed between rheumatoids and normals. While results were not compatible with the existence of an isolated defect in suppressor T cell function, there was a possibility that hyporesponsiveness of the suppressor T cell co-existed with other defects. In order to further evaluate the nature of this defect in immunoregulation, further studies were planned to enumerate cells in the non-adherent T cell preparation and examine enhancement using a different manoeuvre but the same endpoints.

SUMMARY:

A review of the literature suggested that while several immune abnormalities had been described in R.A., relatively little was known regarding regulation of the immune response in this disease. Since defective immunoregulation may account for several observations, interest in this area is more than casual. Several workers, using conconavalin A generated suppressor cells and ovalbumin specific PFC assay systems, have suggested that suppressor T cell activity is abnormal in rheumatoid arthritis. To date, immunoregulation in R.A. has not been systematically examined using the reverse haemolytic plaque forming cell assay and manipulation of lymphocyte subpopulations by optimal dose radiation.

Immunoglobulin synthesis was examined in 13 patients with classical rheumatoid arthritis and 14 normal controls. Responses were similar in rheumatoids and normals for each cell combination (B cells alone, T cells alone, B + non-irradiated T, B + T6400, unseparated lymphocytes), with the exception of cocultures of B cells with T3200 cells, where a failure of radiation enhancement was noted in rheumatoids. There was no evidence that this enhancement failure was related to type of drug therapy, disease duration, disease activity as reflected by the ESR, sex, age, or rheumatoid factor status. The observation that immunoglobulin synthesis was enhanced by coculture with autologous non-irradiated T cells but not further enhance by coculture with T3200 cells suggested several possibilities: (1) decrease in the number or function of suppressor T cells, (2) decrease in the number or function of helper T cells, (3) decrease in the number and/or the function of helper and suppressor T cells, (4) B cells were restricted in their capacity to respond to helper signals, (5) rheumatoid monocytes were reduced in numbers or functions, (6) there was a failure in the release or action of soluble factors or (7) the results were the result of laboratory artefact. The data did not support the presence of an isolated T suppressor cell dysfunction although combined helper and suppressor dysfunction could not be excluded.

While several alternative possibilities required evaluation there was no evidence that the results were artefactual. However, the results of this study indicated a necessity to further evaluate the number and function of involved mononuclear cell subsets and the release and potency of soluble helper factors.

CHAPTER 5

ENUMERATION AND FUNCTIONAL ASSESSMENT OF T LYMPHOCYTES BEARING Fc RECEPTORS FOR IgG (Tγ CELLS)

(STUDY 7)

- INTRODUCTION
- SUBJECTS AND METHODS
- RESULTS
- SUMMARY

"When you cannot measure it, when you cannot measure it in numbers, you have scarcely, in your thoughts, advanced to the stage of science, whatever the matter be."

Lord Kelvin (1824-1907)

INTRODUCTION

The existence of functional lymphocyte subsets has been recognized since the development of heterologous antisera[272]. In the preceding chapter the functional capacity of lymphocytes was observed in a system dependent on the relative radio-sensitivities of differing subsets of lymphocytes. An alternative method of examination is afforded by the recognition that different functional subsets of T cells vary in the types of Fc receptors they express on their cell surfaces[273]. While the presence of immunoglobulin receptors on T cells has been previously noted in mice[274-278], it is only relatively recently that their presence on human T cells has been demonstrated[279-282]. Only a small proportion (less than 20%) have receptors for IgG (Tγ), while up to 75% have receptors for the Fc fragment of IgM (Tu). The remainder lack detectable receptors for either IgG or IgM. Fractionating T cells with Fc receptors by their capacity to bind IgG or IgM immune complexes, allows dissection of their function in in vitro assay systems[273, 283, 284]. Experiments conducted on normal human peripheral lymphocytes[273] have demonstrated that while T cells pre-irradiated at 3,000 rads are fully capable of providing B cell help (intact T helper function), pre-irradiation of Tγ cells (3,000 rads) results in a loss of suppression in a normal differentiation system. Thus it appears from a functional standpoint that

in normals the radiosensitive T cell and the Tγ cell show a common function and that these two manoeuvres manipulate different facets of the same cell. This relationship has not previously been examined in rheumatoids.

Attention has been drawn to the effect of technical manipulation on the expression and avidity of Fc-IgG receptors on Tγ cells[285]. It has been noted that Fc-IgG receptors increase after cell activation[276] and decrease after cells are exposed to immune complexes[286, 287]. Furthermore, 5 to 15% more lymphocytes have have been detected in isolated E-rosetted preparations than in unseparated lymphocytes[285], and Ficoll-Hypaque has the ability to induce B cells to generate lymphokines[288]. These studies indicate that the procedures used to isolate T cells may result in activation and alteration of receptor expression and avidity. Moreover, low avidity Fc-IgG receptors may be expressed on B cells, and high avidity Fc-IgG receptors on L cells (non B non T populations)[285], a fact which may result in enumeration errors where there is a significant non-T cell contamination. Finally, Pickeler and Broder[289] have described a transition of Tγ cells to Tu cells (and vice versa) and postulated that Fc-IgG and Fc-IgM receptors may not be markers for distinct T cell subsets, but that certain T cells may express both classes of receptors at different functional stages. Using T lymphocytes from 11 healthy

volunteers, they studied Fc-IgG and Fc-IgM receptor expression by a rosette technique employing bovine red cells coated with rabbit IgG (EA-IgG) or IgM (EA-IgM). Their results indicate that (1) most T cells have either Fc-IgG or Fc-IgM receptors, (2) that some Tγ cells lose their Fc-IgG receptors spontaneously in culture at 37°C, (3) that most Tγ cells lose their Fc-IgG receptors after immune complex interaction, (4) that Tγ cells acquire Fc-IgM receptors in culture after immune complex interaction, and (5) that simultaneous expression of both Fc-IgG and Fc-IgM receptors is seen under certain conditions (since the sum of Fc-IgG + and Fc-IgM + cells occasionally exceeded 100%).

Brochier et al[290] developed a rosette technique using chicken red blood cells coated with rabbit anti-chicken erythrocyte antibodies, to identify human mononuclear cells bearing Fc receptors. Since intact 7S IgG but not their F (ab_2) fragments were found to sensitise chicken erythrocytes, it was concluded that cells bearing receptors for the Fc portion of IgG were being detected. While it appears that rosetting is not a feature characteristic of any one subclass of lymphocytes, B cell and monocyte contamination of high quality non-adherent cell preparations is negligible and therefore cells detected by this method are a predominantly T lymphocyte subset (Tγ cells). Evidence will be presented to demonstrate that depletion of this subset results in an alteration of the help suppression balance.

R.A. Tγ cells have recently been enumerated by Mathieu et al,[291] using ox red blood cells coated with an IgG fraction of antibovine RBC serum. There was no statistically significant difference between the percentage of Tγ cells in the peripheral blood of 10 rheumatoids (10.6 ± 4.6%) and 12 normal subjects (11.7 ± 4%). However, R.A. peripheral blood Tγ cells had a greater number and/or avidity of Fc receptors for IgG, than those of controls. (There were significantly fewer Tγ cells in rheumatoid synovial fluid (3.4 ± 3%) than in controls (10.1 ± 1.6%).

An earlier report by Biberfeld [292], using a slightly different method but still employing coated ox red blood cells, indicated that the peripheral blood Tγ cells in rheumatoids were increased, although only 6 patients were enumerated. The range for normals in this study was also higher: 16 ± 3%.

In the experiments which follow, modification of the method of Brochier et al was used to enumerate Tγ cells in rheumatoids and normals. The effects of Tγ depletion and/or irradiation were compared to judge the functional comparability of lymphocyte subsets manipulated by the two manoeuvres. Finally, an extension of Brochier et al's technique allowed the effects of serial submaximal Tγ depletion on immunoglobulin synthesis by B cells to be studied.

SUBJECTS AND METHODS.

Patients selected for this study all had classic or definite rheumatoid arthritis. Four types of experiments were conducted:

(A) Tγ enumeration.

Enumeration of Tγ cells - 9 patients with rheumatoid arthritis (MW, BF, BL, HM, SS, HB, DB, SW, DM) and seven controls (nb, ke, db, jh, jd, mm, nw) were studied (Appendices 1 and 2).

(B) Irradiation of T and T nonγ cells.

The effect of irradiation of T and T nonγ cells on immunoglobulin synthesis by B cells - 2 rheumatoids (BF, BL) and 2 normals (jh, jd) were evaluated. (Appendices 1 and 2).

(C) Serial depletion of T cells.

The effect of rosette depletion of Tγ cells on immunoglobulin synthesis by B cells - 5 rheumatoids (MW, SS, HM, HB, DB) and 3 normals - (nb, db, ke) were studied. (Appendices 1 and 2).

(D) Combined serial depletion and radiation.

The effect on radiation enhancement of serial pre-depletion of Tγ cells - 2 normals (db, ke) were studied. (Appendix 2).

In order to conduct the experiments Tγ cells required identification, separation and in some experiments serial depletion, Enumeration (A) was conducted on T lymphocytes derived from study 6, while in the remaining experiments (B, C, D) lymphocytes were separated from fresh heparanized peripheral blood by the methods previously described.

Enumeration.

Identification and separation of Tγ cells were conducted as follows. Packed chicken erythrocytes (Woodlyn Laboratories Limited, Guelph, Ontario) were examined microscopically on delivery for any morphologic evidence of deterioration. If cells appeared healthy then they were washed three times in B.S.S. Equal volumes of 2% chicken red blood cell (CRBC) suspension in BSS and 1/800 rabbit IgG anti CRBC (Capell Laboratories, Cochraneville, Pennsylvania) were mixed at 37°C with constant agitation on a rocker platform for 30 minutes, and washed three times in 2% v/v bovine serum albumin (BSA) (Grand Island Biological Company, New YOrk, 14072) in phosphate buffered saline (PBS) (ph 7.4) and finally suspended to 0.5% in 2% BSA in PBS. Equal volumes of 0.5% sensitized CRBC and nylon wool separated T cells ($2x10^6$ cells per ml) were incubated at 37°C for 15 minutes, centrifuged at 60g for 5 minutes, incubated at 4°E for one hour and then resuspended. The number of rosette formigg cells was enumerated by counting the number of mononuclear cells with 3 or more adherent chicken red blood cells and expressing as a percent of T cells.

Separation.

Separation of rosetted and non-rosetted (T non γ) cells was effected by underlaying the suspension with Ficoll-Hypaque (Sg 1.079 - 1.082) and centrifuged at 800g for 12 minutes at 4°C. Rosetted

cells were pelleted at the bottom of the tube whilst non-rosetted cells remained above the Ficoll-Hypaque.

Serial Depletion.

Utilizing the above techniques, by varying the volume ratio of sensitized CRBC to unsensitized CRBC, submaximal numbers of rosetted T mononuclear cells were obtained, and thereby T cell populations were serially depleted of Tγ cells. Prior to addition to cultures all cells were washed in RPMI 1640, centrifuged (300g for 8 minutes at 4°C) and resuspended in culture medium.

Four experiments were conducted in the following manner;

A. Tγ cells were enumerated at the same time as conducting functional assessment in Study 6.

B.* This experiment employed all the techniques used in Study 6, but cell combinations differed in their composition. $5x10^4$ B cells and $5x10^4$ T cells were still cultured in 1:1 ratio combinations for seven days as described. However, additional combinations were established of B cells with T cells which had been completely depleted of Tγ cells (=T non γ), and were then either non irradiated or irradiated at 3200 or 6400 rads prior to coculture. The T non γ purity of these preparations was checked by attempting to rerosette some samples. Failure of any further rosette formation, was taken to indicate that

Tγ cells have been effectively removed.

C*. In this experiment, 1:1 ratio combination cultures of $5x10^4$ B cells and $5x10^4$ T cells were established. In contrast to Study 6, radiation was not employed but instead the proportion of suppressor cells (Tγ cells), in the T cell fraction was varied by the rosette depletion method described. Thereafter cells were cultured and immunoglobulin synthesis measured by the reverse haemolytic plaque forming cell assay (see Study 6).

D*. In the final experiment T cell suspensions were serially depleted of Tγ cells and the remaining population of lymphocytes either non irradiated or irradiated at 3200 rads prior to coculture with B cells (1:1 ratio combination) as previously described. Enhancement factors were determined for pairs (B + T3200 + PWM, B + T + PWM) of cultures at each level of Tγ depletion between 0 and 100%.

(* = PWM Lot No. 15K0201)

RESULTS. (Original data- Appendix 3- 768-913)

(A) When enumeration of Tγ cells was conducted under light microscopy (rosettes being defined as the presence of three or more chicken erythrocytes clustered around a central human lymphocyte), a range of percentages was defined for normals (8 to 26%) and rheumatoids (7 to 22%) (Table 15). Although the mean percentage was higher for normals (15.2%), it did not differ statistically from the rheumatoid mean (12.9%) (Figure 27). When enhancement factors were plotted against percentages of corresponding Tγ cells for rheumatoids and normals (Figure 28), no correlation was noted. In particular there was no evidence that enhancement factors were low in those patients with small numbers of Tγ cells. (an observation which would have been consistent with radiation having less effect on cell suspensions containing fewer radiosensitive suppressor cells). The distribution of data points in this figure illustrate a difference in enhancement factors between the two groups but a similarity in the percentage of Tγ cells. These data were further evaluated by examining for any within group association of percentage Tγ cells with ESR, age and drug therapy. (Figure 29). Since no seronegative rheumatoids were studied in this fashion no comparison could be drawn between sero-

TABLE 15 - ENUMERATION OF Ty CELLS (SIMULTANEOUS ENHANCEMENT FACTOR DETERMINATION).

NORMAL

	B+T+PWM	$B+T_{3200}$+PWM	E F	%Ty
n.b.	5625	10600	1.9	10
k.e.	1950	16400	8.4	8
d.b.	2500	7900	3.2	26
j.h.	2491	8750	3.5	18
j.d.	2175	4194	1.9	15
m.m.	3428	8228	2.4	12
b.w.	1676	2631	1.6	18

MEAN %Ty = 15.2%

R.A.

	B+T+PWM	$B+T_{3200}$+PWM	E F	%Ty
M.W.	6600	5000	0.76	22
B.F.	1450	1700	1.2	8
B.L.	1200	1500	1.3	8
H.M.	2247	2210	0.98	7
S.S.	1232	1247	1.01	9
H.B.	2160	1833	0.85	20
D.B.	2460	3037	1.2	15
S.W.	4090	3718	0.91	15
D.M.	3159	3785	1.2	12

MEAN %Ty = 12.9%

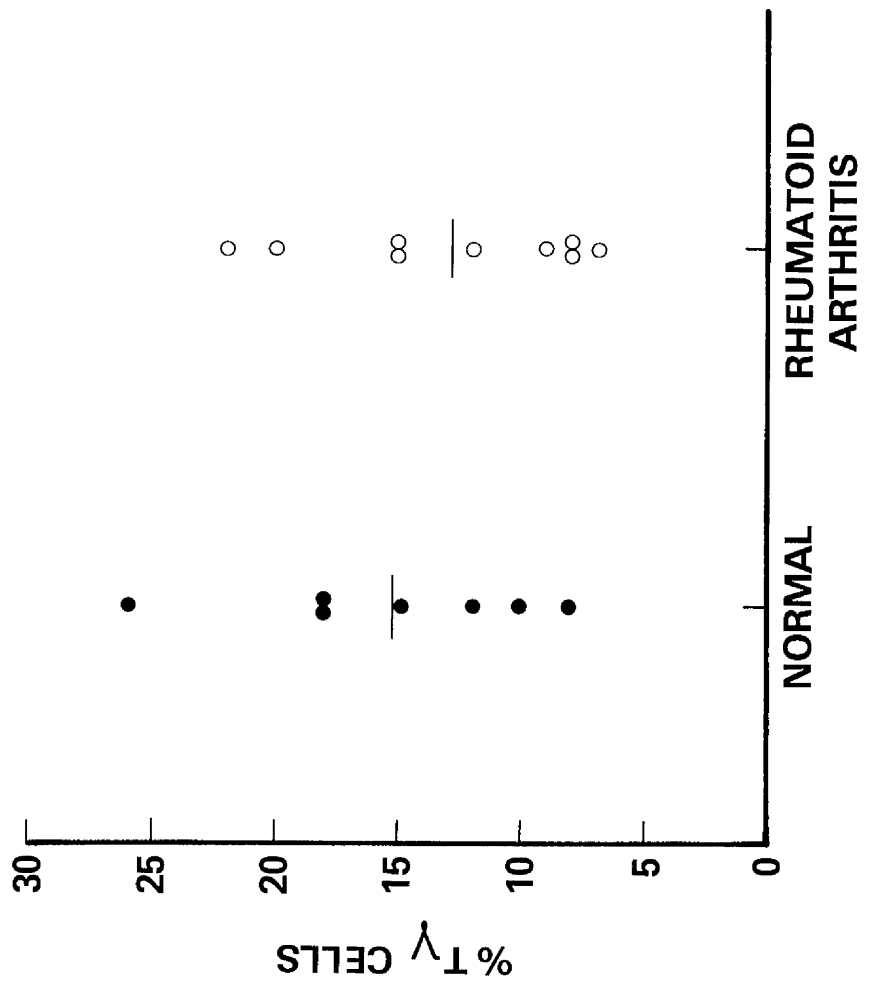


FIGURE 27 - Tγ ENUMERATION IN R.A. AND NORMAL SUBJECTS.

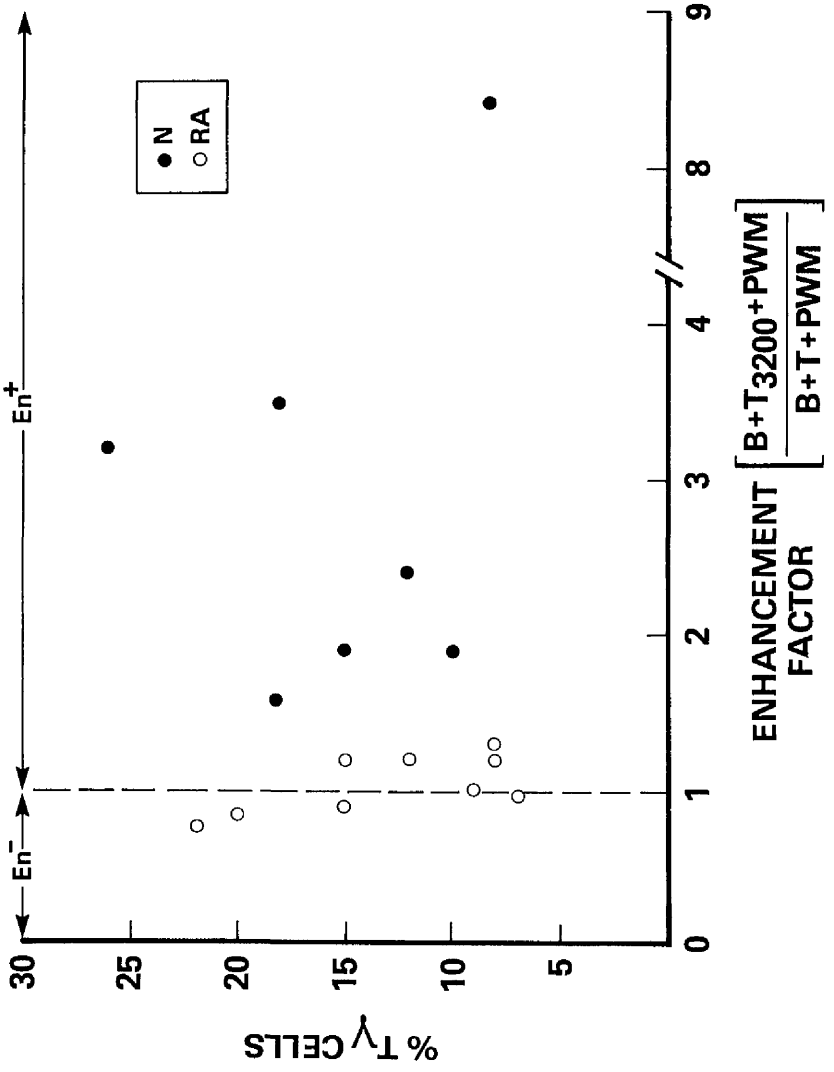


FIGURE 28 - RELATIONSHIP BETWEEN %Tγ and ENHANCEMENT FACTOR IN R.A. AND NORMAL.

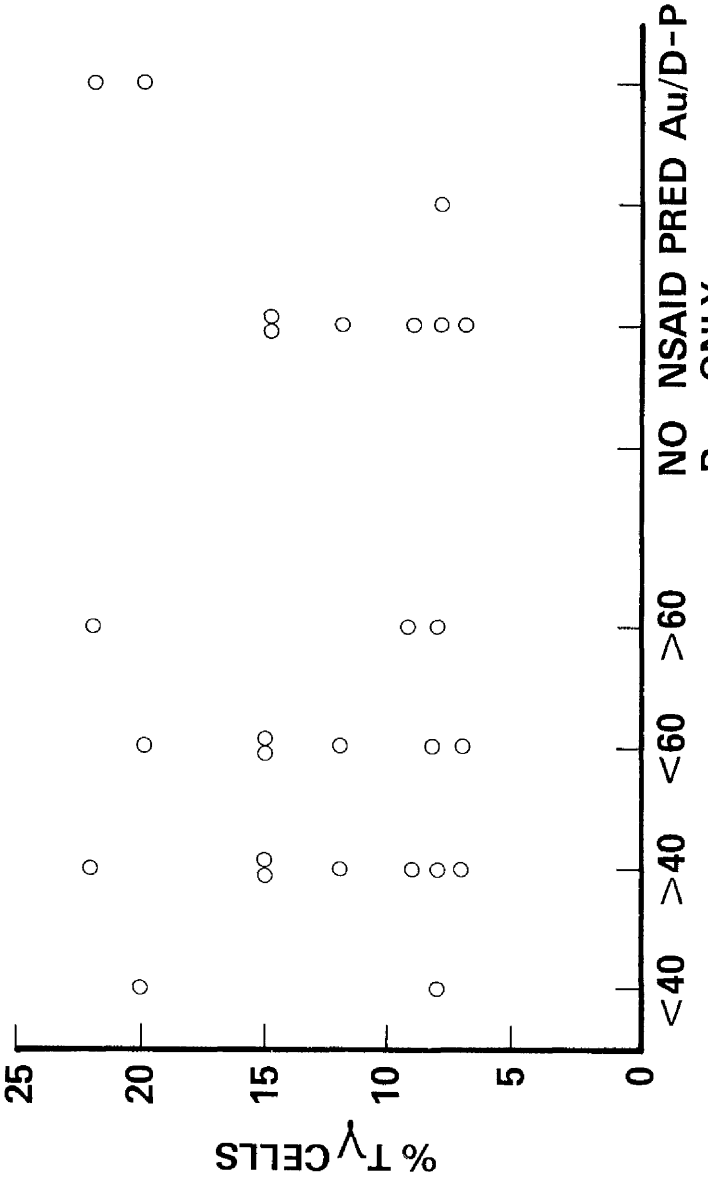


FIGURE 29 - DISTRIBUTION OF $T\gamma$ WITH ESR, AGE AND DRUG THERAPY IN R.A.

positive and seronegative patients. It was evident that neither disease activity as measured by ESR nor age correlated with the percentage of Tγ cells in any individual. Although Tγ percentages were slightly higher in two patients on remittive agents, no significance can be drawn because of the small numbers involved. It was evident that within the non-steroidal anti-inflammatory only group of patients, there existed a range for %Tγ

(B) Irradiation of T and T non γ cells.

In this experiment, part of which was reported in Chapter 4, B and T cell separation was effective as indicated by a failure of the plaque response in preparations of T cells alone, and low responses in preparations of B cells alone (Table 16). When non-irradiated T cells were added to B cells, enhancement was noted in both rheumatoids (1687, 1037) and normals (2491, 2175) As reported radiation enhancement was observed in normals (8750, 4194) but not in the patients with rheumatoid arthritis (1665, 1332). When T cells were pre-irradiated at 6,400 rads there was decline in the level of the observed response. Responses differed somewhat in that in one case (BF) the decline was to below the level observed from B cells alone, in one (jh) to a level between that observed from B+ non-irradiated

TABLE 16 - IMMUNOGLOBULIN SYNTHESIS IN R.A. AND NORMAL CULTURES CONTAINING IRRADIATED AND NON-IRRADIATED T AND T NON$_\gamma$ CELLS.

PFC/10^6 PBL

CELLS	PWM	R.A. B.L.	R.A. B.F.	NORMAL j.h.	NORMAL j.d.
T	+	0	0	0	0
	-	0	0	0	0
T nonγ_{3200}	+	--	0	--	-
	-	-	0	--	-
B + T	+	1687	1037	2491	2175
	-	-	473	750	264
B + T nonγ	+	1765	1332	7768	5250
	-	-	475	2591	1750
B + T$_{3200}$	+	1665	1332	8750	4194
	-	-	392	2600	600
B + T nonγ_{3200}	+	1775	1450	8797	5650
	-	-	996	2706	2000
B + T$_{6400}$	+	-	90	3000	1040
	-	-	425	330	132
B + T nonγ_{6400}	+	561	637	2925	1725
	-	-	315	800	450
B	+	199	280	643	200
	-	-	100	100	50
U	+	-	-	3844	-
	-	-	-	742	-
%T$_\gamma$		8	8	18	15

U = Unseparated lymphocytes

PWM Lot # = 15K0201

Original data for jh and jd in Appendix 3 (cultures 434-489).

Responses for BL and BF represent single determinations.

T cells and B + T 3,200 cells, and in the final case (jd) was intermediate between that observed from B cells alone and B + non-irradiated T cells, a response more consistent with that seen in pooled data. Three important observations were made with this experiment (Figure 30). Firstly, it was noted that enhancement of immunoglobulin synthesis could be achieved in normals by the removal of Tγ cells prior to coculture with B cells and that this enhancement (within the variability of the test) was of the same order of magnitude as that achieved with pre-irradiation of T cells at 3,200 rads. While rheumatoid B cells responded to coculture with non-irradiated T cells, no further enhancement was noted with either irradiation or with coculture with T non γ cells. Secondly, when T non γ cells were irradiated at 3,200 rads prior to coculture with B cells observed responses in rheumatoids and normals were only slightly greater than those observed with non-irradiated T non γ cells. From the original data table it was evident that in one normal (jh) and both rheumatoids (BL, BF) those differences could be accounted for by variation in observed responses from replicate determinations, while in the other normal (jd) it was slightly outwith test variation. Finally, in rheumatoids responses from B + T, B + T_{3200}, B + T nonγ and B + T $nonγ_{3200}$ combinations (+PWM) were similar.

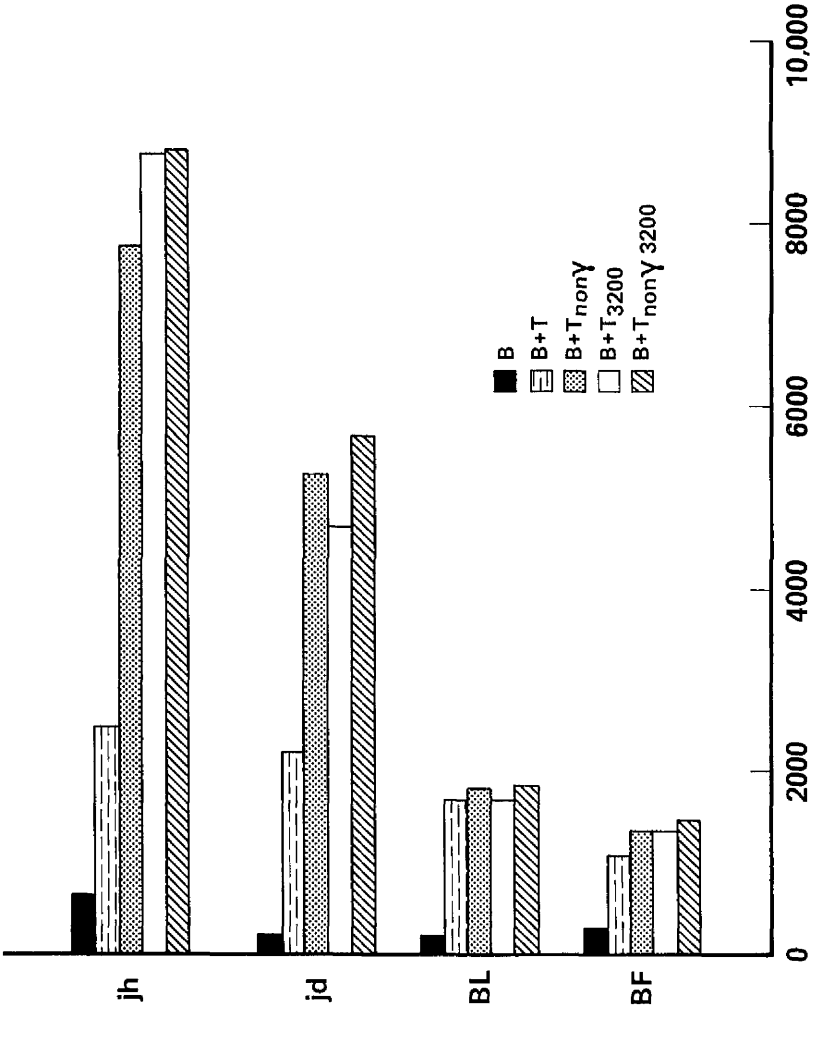


FIGURE 30 - IMMUNOGLOBULIN SYNTHESIS IN CULTURES CONTAINING IRRADIATED AND NON IRRADIATED T AND T NON γ CELLS IN TWO RHEUMATOIDS (BL, BF) AND TWO NORMAL SUBJECTS (jh, jd). ALL CELLS CULTURED WITH 1% PWM.

(C), Serial depletion of Tγ cells.

Effective cell separation was demonstrated in these experiments by the absence of immunoglobulin synthesis in cultures containing T cells alone, and low responses in cultures containing B cells alone. Pokeweed mitogen consistently enhanced the observed response, as did coculture of B cells with non-irradiated T cells. (Tables 17-24). In order to illustrate the method of assessing the degree of Tγ depletion, the calculation used for normal subject (db) will be illustrated. When the sensitized to unsensitized CRBC ratio was 0:1 (B+T_4 + PWM), no rosettes formed and therefore the number of Tγ cells remaining was designated 100 per cent. Since no change had been effected in the T cell population results approximated those in B+T+PWM cultures (1931 versus 2,000). Thus these results were averaged to provide a mean response representative of the presence of an intact Tγ cell population. When the sensitized to unsensitized CRBC ratio was 1:0 (B+T+PWM) a maximum number of rosettes formed (checked by the absence of rosette formation on attempted re-rosetting), and that percentage of rosettes (26%), was equivalent to 100 per cent depletion. When an intermediate reduction in the volume proportion of sensitized CRBCs (1:2) was made (B+$T_{\frac{1}{2}}$+PWM), less rosettes formed (14%). Since 26% was the maximum number of rosettes obtainable, 14% was 14 ÷ 26 or 53% of total.

TABLE 17 - SERIAL T_Y DEPLETION IN NORMAL SUBJECT (n.b.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%T_Y REMOVED	%T_Y REMAINING	PFC/10^6PBL
n.b.	B+PWM	-	-	-	-	250
	B-PWM	-	-	-	-	100
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	4575
	B+T-PWM	-	-	-	-	2400
	B+T_1+PWM	1:0	11	100	0	8325
	B+T_1-PWM	1:0	11	100	0	3050
	B+T_2+PWM	1:2	6	54	46	5825
	B+T_2-PWM	1:2	6	54	46	2700
	B+T_3+PWM	1:4	2	18	82	5375
	B+T_3-PWM	1:4	2	18	82	2450
	B+T_4+PWM	1:8	0	0	100	5250
	B+T_4-PWM	1:8	0	0	100	2050

* S/U = Sensitised: Unsensitised

TABLE 18 - SERIAL T_γ DEPLETION IN NORMAL SUBJECT (d.b.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%$R\gamma$ REMOVED	%$R\gamma$ REMAINING	PFC/10^6PBL
d.b.	B+PWM	-	-	-	-	425
	B-PWM	-	-	-	-	190
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	2000
	B+T-PWM	-	-	-	-	800
	B+T_{3200}+PWM	-	-	-	-	6700
	B+T_{3200}-PWM	-	-	-	-	1500
	B+T_{6400}+PWM	-	-	-	-	800
	B+T_{6400}-PWM**	-	-	-	-	390
	B+T_1+PWM	1:0	26	100	0	6880
	B+T_1-PWM	1:0	26	100	0	1332
	B+$T_{1_{3200}}$+PWM	1:0	26	100	0	7200

*S/U = Sensitised: Unsensitised

TABLE 18 - SERIAL TY DEPLETION IN NORMAL SUBJECT (d.b.)

CONTD.

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%TY REMOVED	%TY REMAINING	PFC/10^6PBL
db	B+T_1 -PWM	1:0	26	100	0	1100
	B+$T_{1_{3200}}$ -PWM					
	B+T_2+PWM	1:2	14	53	47	5100
	B+T_2-PWM	1:2	14	53	47	800
	B+$T_{2_{3200}}$ +PWM	1:2	14	53	47	5800
	B+$T_{2_{3200}}$ -PWM	1:2	14	53	47	1300
	B+T_3+PWM	1:4	10	38	62	4295
	B+T_3-PWM	1:4	10	38	62	600
	B+$T_{3_{3200}}$ +PWM	1:4	10	38	62	5150
	B+$T_{3_{3200}}$ -PWM	1:4	10	38	62	900
	B+T_4+PWM	0:1	0	0	100	1931
	B+T_4-PWM	0:1	0	0	100	800
	B+$T_{4_{3200}}$ +PWM	0:1	0	0	100	5300
	B+$T_{4_{3200}}$ -PWM	0:1	0	0	100	1300

*S/U = Sensitised: Unsensitised

TABLE 19 - SERIAL Tγ DEPLETION IN NORMAL SUBJECT (k.e.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%Tγ REMOVED	%Tγ REMAINING	PFC/10^6PBL
k.e.	B+PWM	-	-	-	-	135
	T+PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	2204
	B+T_{3200}+PWM	-	-	-	-	9019
	B+T_{6400}+PWM	-	-	-	-	1220
	B+T_1+PWM	1:0	8	100	0	7472
	B+$T_{1_{3200}}$+PWM	1:0	8	100	0	8859
	B+T_2+PWM	1:2	3	37	63	6637
	B+$T_{2_{3200}}$+PWM	1:2	3	37	63	7859
	B+T_3+PWM	1:4	1	12	88	4320
	B+$T_{3_{3200}}$+PWM	1:4	1	12	88	8118
	B+T_4+PWM	0:1	0	0	100	2028
	B+$T_{4_{3200}}$+PWM	0:1	0	0	100	6608

*S/U = Sensitised: Unsensitised

TABLE 20 - SERIAL T_γ DEPLETION IN RHEUMATOID SUBJECT (M.W.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%Tγ REMOVED	%Tγ REMAINING	PFC10^6
M.W.	B+PWM	-	-	-	-	650
	B-PWM	-	-	-	-	150
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	4600
	B+T-PWM	-	-	-	-	999
	B+T_1+PWM	1:0	22	100	0	3650
	B+T_1-PWM	1:0	22	100	0	1750
	B+T_2+PWM	1:2	13	41	59	3500
	B+T_2-PWM	1:2	13	41	59	800
	B+T_3+PWM	0:1	0	0	100	3250
	B+T_3-PWM	0:1	0	0	100	1000

*S/U = Sensitised: Unsensitised

TABLE 21 - SERIAL T_γ DEPLETION IN RHEUMATOID SUBJECT (S.S.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%T_γ REMOVED	%T_γ REMAINING	PFC/10^6PBL
S.S.	B+PWM	-	-	-	-	125
	B-PWM	-	-	-	-	0
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	2171
	B+T-PWM	-	-	-	-	938
	B+T_1+PWM	1:0	9	100	0	2249
	B+T_1-PWM	1:0	9	100	0	688
	B+T_2+PWM	1:2	8	88	12	2281
	B+T_2-PWM	1:2	8	88	12	750
	B+T_3+PWM	1:4	4	44	56	2125
	B+T_3-PWM	1:4	4	44	56	563
	B+T_4+PWM	0:1	0	0	100	2495
	B+T_4-PWM	0:1	0	0	100	813

*S/U = Sensitised: Unsensitised

TABLE 22 - SERIAL TY DEPLETION IN RHEUMATOID SUBJECT (H.M.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%TY REMOVED	%TY REMAINING	PFC/10^6PBL
H.M.	B+PWM	-	-	-	-	286
	B-PWM	-	-	-	-	0
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	2593
	B+T-PWM	-	-	-	-	1000
	B+T_1+PWM	1:0	7	100	0	2893
	B+T_1-PWM	1:0	7	100	0	1000
	B+T_2+PWM	1:2	6	85	15	2357
	B+T_2-PWM	1:2	6	85	15	929
	B+T_3+PWM	1:4	1	14	86	2460
	B+T_3-PWM	1:4	1	14	86	1071
	B+T_4+PWM	0:1	0	0	100	2404
	B+T_4-PWM	0:1	0	0	100	1500

*S/U = Sensitised: Unsensitised

TABLE 23 - SERIAL T_γ DEPLETION IN RHEUMATOID SUBJECT (H.B.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%T_γ REMOVED	%T_γ REMAINING	PFC/10^6 PBL
H.B.	B+PWM	-	-	-	-	500
	B-PWM	-	-	-	-	100
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	3048
	B+T-PWM	-	-	-	-	1400
	B+T_1+PWM	1:0	20	100	0	5200
	B+T_1-PWM	1:0	20	100	0	1050
	B+T_2+PWM	1:2	9	45	55	4650
	B+T_2-PWM	1:2	9	45	55	1100
	B+T_3+PWM	1:4	4	20	80	3550
	B+T_3-PWM	1:4	4	20	80	1100
	B+T_4+PWM	1:8	0	0	100	2950
	B+T_4-PWM	1:8	0	0	100	1050

*S/U = Sensitised: Unsensitised.

TABLE 24 - SERIAL Tγ DEPLETION IN RHEUMATOID SUBJECT (D.B.)

	CELLS CULTURED	S/U*	% CELLS ROSETTED	%Tγ REMOVED	%Tγ REMAINING	PFC/10^6PSL
D.B.	B+PWM	-	-	-	-	400
	B-PWM	-	-	-	-	200
	T+PWM	-	-	-	-	0
	T-PWM	-	-	-	-	0
	B+T+PWM	-	-	-	-	2500
	B+T-PWM	-	-	-	-	550
	B+T_1+PWM	1:0	15	100	0	2850
	B+T_1-PWM	1:0	15	100	0	600
	B+T_2+PWM	1:2	4	26	74	2425
	B+T_2-PWM	1:2	4	26	74	500
	B+T_3+PWM	1:4	3	20	80	2725
	B+T_3-PWM	1:4	3	20	80	550
	B+T_4+PWM	1:8	0	0	100	2200
	B+T_4-PWM	1:8	0	0	100	500

*S/U = Sensitised: Unsensitised

Therefore 100-53 or 47% Tγ cells remained. A further reduction ($B+T_3$+ PWM) in the volume proportion of sensitized CRBCs (1:4) resulted in even less rosette formation (10%), and by subtraction a greater proportion of unrosetted Tγ cells (62%) remained.

When the Tγ depleted T lymphocyte populations were cultured (as previously described) in a 1:1 ratio with B cells (+PWM), the resulting PFC responses reflected the effects of serial depletion of Tγ cells (Figure 31). With the initial addition of T cells (100% Tγ remaining) B cell responses were enhanced to an equivalent degree in rheumatoids and normals. However, with subsequent serial Tγ depletion, responses differed between the two groups. In normals there was progressive enhancement of the B cell response as Tγ (suppressor) cells were removed, indicating a shift in the help/suppression balance in favour of help. However, in rheumatoids (with one exception) there was no such enhancement as Tγ cells were serially depleted. When Tγ cells were completely depleted PFC responses in normals were greater than 6,000 PFC per 10^6 PBL, while in rheumatoids they were below 6,000 PFC per 10^6 PBL, and with one exception below 4,000 PFC per 10^6 PBL.

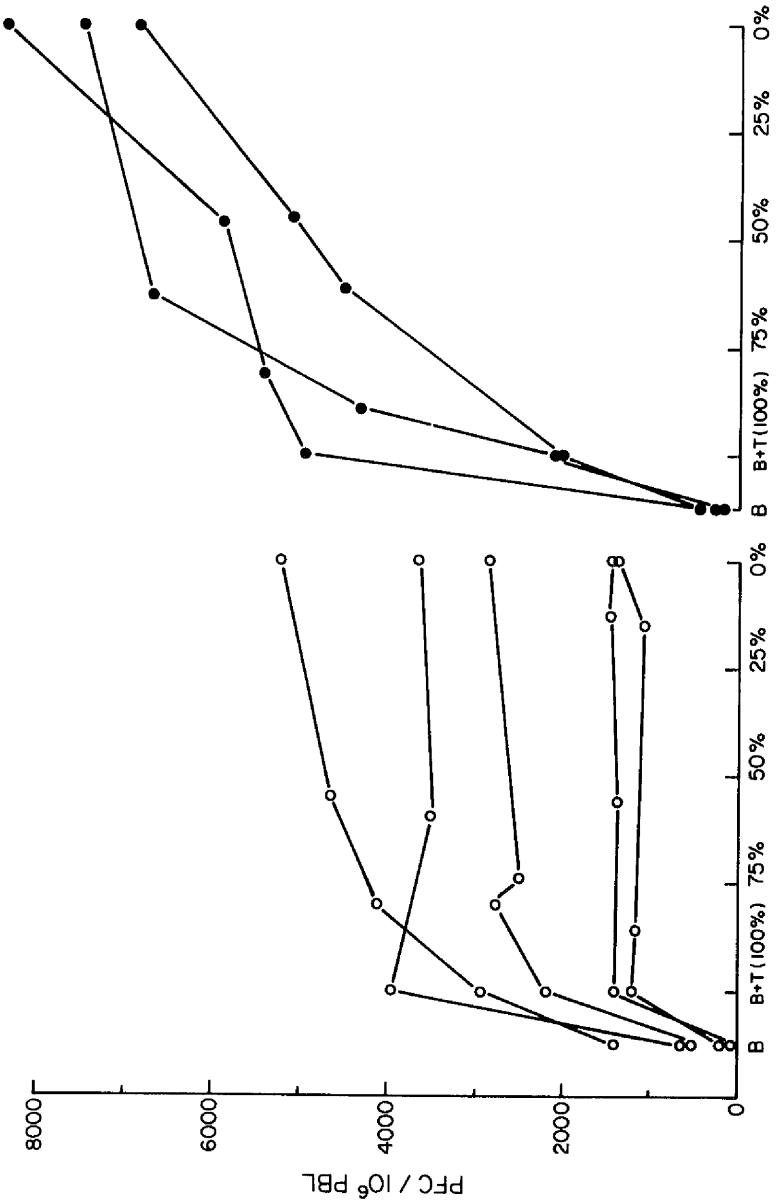


FIGURE 31 - Immunoglobulin synthesis in normal ( ) and R.A.(O) cultures serially depleted of Tγ cells.

Examination of equivalent PWM deficient cultures, indicated that this enhancing effect of Tγ depletion was not observable, and that curves between 0 and 100% depletion were essentially flat in both groups. This indicated that PWM activation of lymphocytes was necessary to detect between group differences.

(D) Combined serial depletion and radiation.

In two patients in whom pairs of T cell suspensions were either non-irradiated or irradiated at 3,200 rads, following varying degrees of Tγ removal (¬Tn) the effect of radiation over and above Tγ removal was observed (Table 18.19). It was evident that, when no Tγ cells remained, no additional enhancement could be achieved by irradiating the remaining T non γ population (as had been previously observed) (Figure 32). When the Tγ population was completely intact the enhancing effect of radiating the T cell suspension was maximal (greater than 3), in accordance with previous observations. Moreover, when submaximal numbers of Tγ cells were depleted, irradiation of the remaining population resulted in degrees of radiation enhancement, proportionate to the number of remaining unrosetted Tγ cells. Thus when few Tγ cells remained in the cell suspension radiation enhancement was low, and when a large number of Tγ cells remained, radiation enhancement was high.

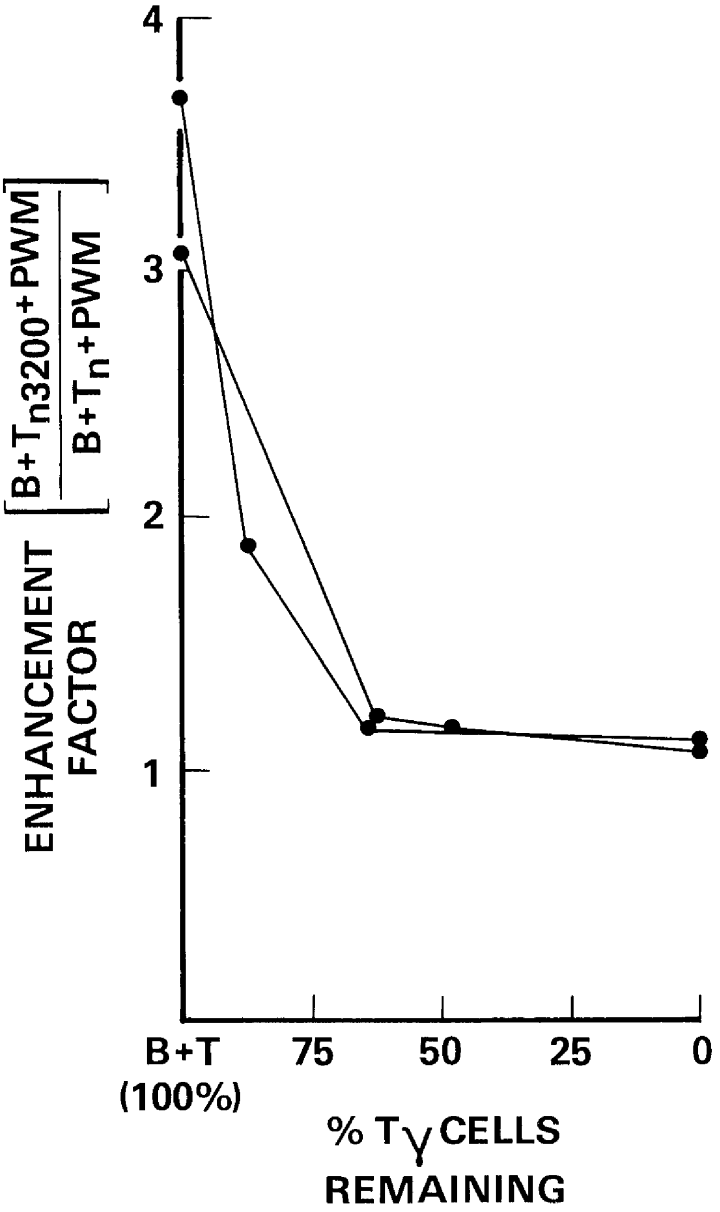


FIGURE 32 - EFFECT OF SERIAL DEPLETION OF T_γ CELLS ON ENHANCEMENT FACTOR IN TWO NORMAL SUBJECTS.

(Tn = T cell suppressor containing a reduced number (n) of T_γ cells).

DISCUSSION.

It was evident that the CRBC rosetting technique was capable of defining a range of normal for the proportion of Tγ cells and could be utilised in manipulating T cell populations, to allow detection of differences existing between rheumatoids and normals. The initial enumeration experiment indicated that the proportion of Tγ cells was similar in the two groups. In particular no deficit or excess of Tγ cells in rheumatoids was detected. Since no relationship was shown between the proportion of Tγ cells and enhancement factors in either rheumatoids or normals it was reasonable to conclude that the radiation enhancement failure in rheumatoids could not be accounted for by a numerical deficiency of cells having suppressor activity. Had radiosensitive Tγ cells been present in reduced numbers in rheumatoid T cell preparations, a failure of alteration in the help suppressor balance with irradiation would have been anticipated. This, however, was not the case and excluded numerical but not functional abnormalities of Tγ cells. Furthermore, this result suggested that the adherance (to nylon wool columns) of R.A. and normal T lymphocytes was similar and that the laboratory artefact referred to in the last chapter was not present. Within the range of Tγ cells for rheumatoids, no association was shown between any particular proportion of Tγ cells and age, disease activity as measured by ESR or drug therapy.

From the second experiment, it was evident, that equivalent degrees of enhancement in normals and non-enhancement in rheumatoids could be achieved by pre-irradiating T cells at 3,200 rads, as by complete Tγ depletion prior to coculture with B cells. This suggested that the radiosensitive cell and the Tγ cell were likely the same cell. Since no additional enhancement over B + T non γ was noted when T non γ cells were also irradiated at 3,200 rads prior to coculture, this suggestion was strengthened. In one instance a slight increase (considered to be outwith test variability) in the PFC response was noted, suggesting the possibilities of radiosensitive T non γ suppressor cells, incomplete removal of radiosensitive Tγ cells or underestimation of the variation in the test. In any event numbers were too small to allow conclusion to be drawn, although the last possibility was most likely. The observation that enhancements in rheumatoids and normals were similar to those in Study 6, and that equivalent responses to T cell irradiation and Tγ depletion were noted within groups, suggested that the physical removal of suppressor cells by Tγ depletion provided a useful alternative method to irradiation in the study of immunoregulation.

In the third experiment, serial Tγ depletion resulted in progressive increase in the PFC response in normals, indicating a definite suppressor role for the Tγ cell. Since enhancement was not observed in rheumatoids who were known to have normal numbers

of Tγ cells, it was suggested either that these cells were hypofunctional, or that T helper activity or B cell responsiveness were limited, or that more than one form of cell dysfunction was in evidence. Pokeweed mitogen was necessary for the identification of between group differences, indicating a necessity for lymphocyte activation.

Finally, in support of previous evidence, observations in two normal subjects, that the less the degree of preculture Tγ depletion, the greater the degree of radiation enhancement of the PFC response (when the partially depleted T cell suspensions were also irradiated at 3,200 rads prior to coculture) indicated that the radiosensitive cell and the Tγ cell had functional similarity and were most probably the same cell.

A unifying concept would be that the great majority of suppressor T cells are both radiosensitive (at 3,200 rads in this laboratory) and also bear Fc receptors for the Fc portion if IgG on their surface. However, a minority of such suppressor cells may be radiosensitive T nonγ cells.

Therefore, in accordance with the finding of Mathieu et al, normal numbers of Tγ cells were present in rheumatoid peripheral blood. The suppressor activity of this functional subpopulation was confirmed and the possibility of radiation enhancement failure in R.A. due to a differential nylon wool adherance artefact

or numerical deficiency of Tγ cells excluded. The significance of restricted in vitro immunoglobulin synthesis by R.A. B cells was strengthened by confirmatory observations using a different method. Thus having excluded a reduction in the number of suppressor T cells and laboratory artefact as explaining the defect, attention was next directed to enumerating monocytes Ia+ and OKT4+ and OKT5+ cells.

SUMMARY.

The CRBC rosette assay detected cells($T\gamma$) whose net functional activity was that of suppression. No differences existed in the proportion of $T\gamma$ cells in rheumatoids and normals. While T3,200 and T nonγ cells enhanced the PFC response from B cells in controls (over and above that of coculture with non-irradiated T cells), no such additional enhancement was observed in rheumatoids. Furthermore, the results of irradiation of T nonγ cells and irradiation of T cell suspensions partially depleted of T cells in normals suggested that the $T\gamma$ suppressor cells was also the radiosensitive suppressor cell. The failure of enhancement noted in rheumatoids may have been due to restricted B cell function, T helper cell hypofunction, or combined T helper and T suppressor cell hypofunction. Since $T\gamma$ cells were known to be present in normal numbers in rheumatoids and the response of B + T + PWM cultures in Study 6 were the same in both controls and patients, it was unlikely that the problem was one of isolated T suppressor cell hypofunction. Finally, in order to observe enhancement in the PFC response, not only must T suppression be reduced (by radiation or $T\gamma$ depletion) but there must also be the facility for B cells to increase immunoglobulin synthesis in response to T helper signals.

CHAPTER 6

MONOCYTE ENUMERATION

(STUDY 8)

- INTRODUCTION
- PATIENTS AND METHODS
- RESULTS
- DISCUSSION
- SUMMARY

"..... in time, the amount of knowledge needed before a new discovery could be made would become so great as to absorb all the best years of a scientists life, so that by the time he reached the frontier of knowledge he would be senile."

Bertrand Russel
"The Science to Save us from Science"
The New York Times Magazine 1950.

MONOCYTE ENUMERATION

INTRODUCTION.

The important role of the monocyte/macrophage in the immune response has been described in Chapter I. Progenitor stem cells differentiate into monoblasts, then promonocytes and finally into monocytes bearing Fc and complement receptors. Mature monocytes are released into the peripheral blood where they circulate and eventually seed different tissues, where they differentiate into tissue macrophages. The circulatory monocyte (constituting 3-8% of blood leucocytes and having a circulatory half-life of 8-24 hours) is 10-18 μm in diameter, has a large centrally located nucleus and in Wright-stained preparations an abundant greyish blue cytoplasm containing small azurophilic granules[293]. Circulating monocytes together with tissue macrophages (histiocytes, Kupffer cells, alveolar macrophages, sinusoidal lining cells, Type A synovial cells, peritoneal macrophages, osteoclasts and microglia), constitute the mononuclear phagocyte system (MPS). The "normal" macrophages may transform into inflammatory macrophages (activated macrophages, epithelial cells and giant cells) when given appropriate signals.

Peripheral blood monocyte counts estimate the number of circulatory monocytes (which may show low amplitude oscillation),

but does not account for the marginated pool which may constitute up to 75% of the total. An increase in the number of circulating monocytes has been described in various conditions, e.g. tuberculosis, syphilis, typhoid, rheumatic fever, SLE, R.A., neoplastic disease, ulcerative colitis and Crohns, post splenectomy, cirrhosis, and sarcoidosis etc. and is common in neonates. An increase in the number of monocytes may be achieved by demargination and/or increased production, the latter (in mice) being partly under the control of a thermal labile protein (molecular weight 18,000 - 24,000 daltons) called FIM (Factor Increasing Monocytopoeisis).

Macrophages are capable of endocytosis (pinocytosis and phagocytosis), and the intracellular digestion of particulate matter. They possess surface receptors for IgG_1 and IgG_3 but not IgG_2 and IgG_4 (there being some evidence for IgM receptors under certain conditions) and for C_{3b} and C_{3d} as well as nonspecific particle receptors. In addition to its phagocytic role the macrophage is also secretory, releasing several enzymes and biologically active factors, some having regulatory activity.

Once a monocyte arrives at a site of inflammation (e.g. R.A. synovium) it undergoes a number of morphologic, metabolic and functional changes in becoming an activated macrophage.

A. Structural
   (1) Larger
   (2) More mitochondria and lysosomes
   (3) More cytoplasmic vesicles
   (4) Increased membrane ruffling

B. Metabolic
   (1) Increased incorporation of glucosamine
   (2) Increased hexose monophosphate shunt activity
   (3) Increased respiratory rate
   (4) Alterations in various enzymes
      (a) Increased content of acid hydrolases
      (b) Increased content of lactate dehydrogenase
      (c) Decrease in plasma membrane 5' nucleotidase
      (d) Increase in plasma membrane adenylate cyclase
      (e) Synthesis and secretion of neutral proteases

C. Functional
   (1) Accentuated glass adherence
   (2) Increased pinocytic rate
   (3) Increased phagocytic capacity
   (4) Augmented ability to kill certain microorganisms
   (5) Augmented tumoricidal capacity.

As noted earlier there is reasonable evidence to suggest that this cell not only presents antigen to the lymphocyte and thereby initiates the immune response, but is also implicated in determining in part the specificity of that response[102]. Thus Rosenthal et al[102] using two strains of guinea pigs were able to demonstrate that the macrophage participates in the expression

of genetic control of the immune response. When immunized with insulin strain 2 guinea pigs reacted only to the A chain, while strain 13 guinea pigs reacted only to the B chain. However, when F_1 (2x13) hybrids were immunized with insulin and their T cells subsequently isolated, it was observed when these cells were cultured with macrophages from either parent strain, the T cells responded only to the A chain in cultures containing strain 2 macrophages and to only the B chain in cultures containing strain 13 macrophages. Thus it appeared in this system, that the macrophage electively determined the specificity of the immune response.

Unanue[102] has drawn attention to the role of the Ia+ macrophage in the immune inductive stage of an immune response to listeria monocytogenese organisms. In the early part of the response, T cells adhered to activated Ia+ monocytes, while 24 hours later Ia- macrophages were noted to have microbicidal properties. Thus in the listeria model Ia positivity was considered a marker for inducer (helper) activity in macrophages.

It was originally claimed that the PWM driven anti SRBC response was independent of monocytes[45]. This claim was based on the fact that when cell preparations were depleted of monocytes to 1% or less, there was still a substantial PFC response. However, another interpretation of this finding, is that only a very small

number of monocytes are required for the reaction to proceed. Monocytes are commonly depleted by one of two techniques: (a) Adherence technique (b) carbonyl iron magnet technique. The former method relies on the physical property of monocytes to adhere to a glass or perspex surface. However, substantial numbers of B cells are also adherent and therefore if an attempt is made to reduce monocyte contamination to 0% there will be a significant loss of B cells. Thus any observed difference may be attributable to removal of B cells with a particular adherence characteristic, rather than removal of monocytes per se[294]. The carbonyl iron magnet method requires that mononuclear cell suspensions are incubated with carbonyl iron prior to being passed through a magnetic field. Phagocytic cells which have ingested iron are then attracted out of the preparation. Total removal of monocytes is unusual by either method, there being up to 3% contamination of the resultant cell suspensions with the adherence technique, and up to 2% with the carbonyl iron method[45].

An observation[295] which requires further evaluation is the differential effect of partial versus complete monocyte depletion. It has been noted that partial depletion of monocytes results in significant enhancement of B cell differentiation, whereas total depletion results in a failure of differentiation. On the basis

of this evidence the authors postulated the existence of monocyte mediated suppression of the B cell response.

It is evident from the foregoing discussion that the study of monocyte/lymphocyte interaction is hindered by the technical difficulty of obtaining monocyte-free lymphocyte and lymphocyte-free monocyte cell suspensions. There are, however, several methods for quantitating monocytes in peripheral blood. The most commonly used method is based on the morphologic appearance of Romanowsky - stained smears and relies heavily on the skill of the observer. Two methods relying on cell function are those of latex bead phagocytosis[296] and cytochemical staining[297]. In the former, cell preparations are incubated with 1.3u latex spherules for one to two hours at 37°C in plastic tubes or fixed to glass surfaces. Monocytes are recognized by their capacity to phagocytose the particles - a process which occurs within 30 minutes in the majority of monocytes. In the latter, cytochemical agents are used to identify intracellular enzymes. I conducted three pilot studies in London comparing both methods and discovered several problems using the latex method. Firstly, granulocytes as well as monocytes phagocytosed latex particles and were often difficult to differentiate because of obscuration of cell morphology by a heavy load of latex spherules. More importantly latex particles adhered to the surface of many cells and it was not possible to ascertain whether spherules had been truly phagocytosed or were merely adherent to the superior or

inferior surface of the cell. Latex particles were often strongly adherent, and present in an excessive number, necessitating repeated cell washing prior to counting. Finally, results lacked internal consistency when repeated on the same sample and disagreed with results obtained simultaneously with cytochemical methods which were consistent. For these reasons I rejected latex phagocytosis as an enumeration technique. However, other researchers using thorough washing techniques have found agreement between the two methods, observing five to fifteen per cent monocyte composition in Ficoll-Hypaque purified peripheral blood mononuclear cells[298].

Davis and Ornstein[299] recognized that the coupling of pararosaniline hexazonium chloride with phenolic compounds produced a deep red brown azo dye, which could localize intracellular enzymatic activity and was free from diffusion artefact. This method was subsequently applied in the identification of acid phosphatase using pararosaniline as a coupler, demonstrating a significant advance over previous histochemical methods in that it was simple and reproducable[300]. Yam et al[297] then applied the technique to the evaluation of enzyme activity in monocytes and granulocytes in blood and tissue smears. Nonspecific esterase activity was noted to be very strong in monocytes and megakaryocytes but weak in granulocytes and lymphocytes. Furthermore, activity

was generalized in monocytes but if present at all in lymphocytes was represented by a small red-brown "dot". It was later noted[301] in mouse lymph node preparations that lymphocytes containing this dot were located principally in T lymphocyte areas and were infrequent in predominantly B cell areas, suggesting a new method for differentiating T and B lymphocytes. A subsequent study[302] in humans, showed that amongst Ficoll-triosil separated mononuclear cells, 19.2% had a diffuse pattern, 61.8% had discrete granules and 19.2% no activity. Cell identification techniques suggested that 80% of T cells contained discrete granules and that of the diffusely staining cells 20% were not phagocytic. Moreover, if these cells were incubated for 48 hours then the number of esterase positive cells increased, most of them being phagocytic.

It has been noted that preparations can be stored for two to three weeks without loss of staining properties, and that Ficoll-triosil separated cells have a lower nonspecific esterase activity than unseparated peripheral blood cells. Since cytocentrifugation procedures are common place it has been suggested[203], that prolonged air drying prior to staining and extension of immersion time in hexazonium chloride improves staining characteristics. In respect of the B lymphocyte population, more than 90% show no activity and

the remaining cells show only dots[304]. Evidence from inhibition assays suggest that this staining reaction is dependent on A-esterases. It has been questioned whether the - absence of esterase activity in T lymphocytes is a marker for T suppressor cell function. At the present there is no evidence to indicate that this is the case.

Although in this thesis cell preparations have been referred to as peripheral blood lymphocytes (PBL), they are in reality peripheral blood mononuclear cells (PBM), in that they contain not only lymphocytes but also monocytes. In addition there may be minor contamination with erythrocytes, platelets and granulocytes. One of the suggested explanations for failure of radiation enhancement in rheumatoids is a reduction in the number or function of immunocompetent monocytes, either as a feature of the disease or as a result of cell processing in vitro.

This study was designed to enumerate monocytes in the three cell suspensions used [unseparated lymphocytes, nylon wool passed (T) and nylon wool adherent (B)] in rheumatoids and normals.

PATIENTS AND METHODS.

Thirteen patients with definite rheumatoid arthritis (GW, DN, CS, WS, MS, JC, SW_1, NI, MS, RR, CF, TM) and 12 normal subjects (nb, mf, ac, ds, du, bw, cr, db, cm_1 jo, jd, ce) were chosen for study. (Appendices 1 and 2). Lymphocytes were harvested from 30mls of heparanized fresh peripheral blood by the methods described in Chapter 4. Following removal of an aliquot of unseparated lymphocytes adherent and non-adherent cells were separated using the nylon wool technique described in the same chapter. Following resuspension by slow vortexing a sample of each cell suspension (unseparated, nylon wool adherent (B) and nylon wool passed (T) lymphocytes) was thinly layered by pipette on to the centre line of a glass microscope slide. Each preparation was allowed to dry in air. Slides were stained using a modification of the cytochemical method described by Yam et al[197]. Primary staining was affected with a preparation of hexazonium pararosanaline chloride counter staining with 1% methyl green.

Preparation of stain.

Pararosanaline dye was prepared as follows. Three solutions were required. (a) Phosphate buffer (b) pararosanaline hydrochloride (c) 4% sodium nitrite.

a) 1 litre of phosphate buffer was prepared by dissolving 1.72 grams of KH_2PO_4 and 7.66 grams of Na_2HPO_4 in one litre of distilled water.

(b) Pararosanaline dye was prepared by dissolving 5 grams of pararosanaline hydrochloride (Eastman Company) in 100 mls of distilled water, and 25 mls of concentrated hydrochloric acid whilst maintaining the temperature of reagents at 37°C. The resulting solution was allowed to cool to room temperature and thereafter filtered through a Nalgene vacuum filter.

(c) 25 mls of 4% sodium nitrite were prepared by dissolving 1 gram of sodium nitrite in 25 mls of distilled water.

Thereafter the stain was prepared as follows: 60mgs of alpha naphthalacetate (Eastman Company) were dissolved in 3mls of Ethylene Glycol Monoethyl Ether (Eastman Company). 0.5mls of this solution were added to 44.5 mls of phosphate buffer with constant stirring for 3-15 minutes. The resulting solution was white initially but cleared with continued stirring leaving behind a white precipitate. To this solution was added 3 mls of a solution prepared by adding 2 mls of 4% sodium nitrite to 2 mls of pararosanaline hydrochloride. (These two reagents required mixing for a period of 1 minute to allow hexazotisation prior to addition to the phosphate buffer). The final solution was stirred for a period of 15 seconds. Thereafter the pH was adjusted to 6.1 by

dropwise addition of IN sodium hydroxide. The solution was then filtered through a nalgene vacuum filter and used within a period of 2 hours.

Staining Procedure.

Air dried slides were placed in a staining dish containing 50 mls of pararosanaline dye for a period of 45 minutes. Thereafter the slides were washed with distilled water and allowed to air dry for half an hour. They were then counterstained for 55 seconds in 1% methylgreen, rinsed again with distilled water and allowed to air dry prior to mounting in Permount. Between 100 and 1,000 cells were counted per slide. Monocytes were identified by their size and the presence of deep red-brown colouration (Figure 33). Lymphocytes were smaller in size, took up the methylgreen counterstain and occasionally contained small red-brown dots. Polymorphs also took up the methylgreen stain but were easily identified by their characteristic nuclear morphology. Monocytes were frequently surrounded by several smaller round brown particles which were probably platelets. The number of monocytes present was expressed as a percentage of the total number of mononuclear cells counted in the same field for each preparation.

Results.

Although the intensity of staining in both monocytes and lymphocytes varied it was technically satisfactory in all preparations. It was obvious on initial low power screening that nylon

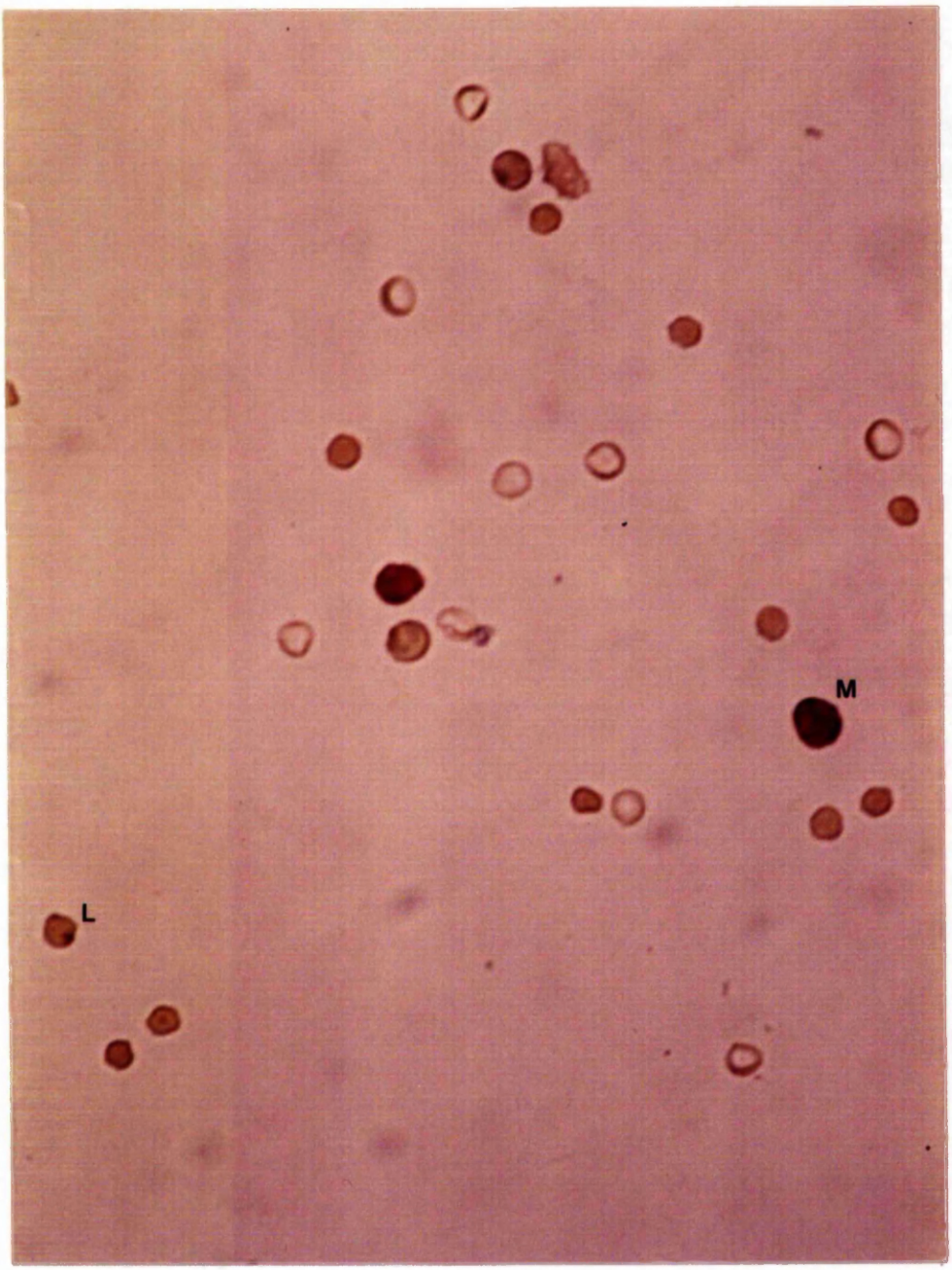


FIGURE 33 - ENUMERATION OF MONOCYTES AND LYMPHOCYTES EMPLOYING NONSPECIFIC ESTERASE STAIN AND METHYLGREEN COUNTERSTAIN (Slide stored for several months prior to photography). MONOCYTES (M) APPEAR AS LARGE RED-BROWN CELLS WHILE LYMPHOCYTES (L) APPEAR AS SMALLER GREEN CELLS. (L) IS A LYMPHOCYTE CONTAINING AN ESTERASE DOT.

wool passed (T) cell preparations were depleted of monocytes. Enumeration of unseparated lymphocyte preparations demonstrated a range of values in both normal subjects (9-31%)(Table 25) and rheumatoids (11-32%) (Table 26). However, means were similar (17.75% and 19.8% respectively), and there was substantial overlap of the standard errors. There was no statistically significant difference between normals and rheumatoids (Figure 34). The percentage of monocytes in nylon wool passed (T) cell preparations ranged 1-4% in normal subjects and 0.5 - 4% in rheumatoids. Similar mean percentages of monocytes were found in normal subjects (1.4 ± 0.3) and rheumatoids 2.1 ± 0.3) although they were substantially less than in unseparated lymphocyte suspensions. Adherent (B) cell preparations from normal subjects and rheumatoids contained similar percentages of monocytes. Although ranges were broad (2.51 and 3.41) mean values were similar (17.83 and 18.1) and standard errors overlapped. There was no statistically significant difference between the two groups. Finally, the percentage of monocytes in B cell and unseparated preparations was similar.

TABLE 25 - ENUMERATION OF MONOCYTES IN NORMAL SUBJECTS.

% MONOCYTES

	CELL FRACTION		
Subject	Unsep	B	T
n.b.	9	2	1
m.f.	16	16	1
a.c.	20	21	1
d.s.	10	17	2
d.v.	15	15	1
b.w.	21	32	1
c.r.	19	9	1
d.b.	15	13	1
$c.m._1$	31	7	1
j.o.	10	11	4
j.d.	21	20	2
c.e.	26	51	1
MEAN	17.75	17.83	1.4
SEM	$\pm$1.9	$\pm$3.7	$\pm$0.3

TABLE 26 - ENUMERATION OF MONOCYTES IN RHEUMATOID SUBJECTS.

% MONOCYTES

CELL FRACTION			
Subject	Unsep	B	T
G.W.	12	20	2
D.N.	22	18	2
C.S.	28	12	4
W.S.	32	22	3
M.S.$_1$	14	7	1
M.B.	18	3	1
J.C.	13	29	1.5
S.W.$_1$	11	4	0.5
N.I.	21	24	3
M.S.	20	16	2
R.R.	25	10	2
C.F.	23	29	3
T.M.	19	41	2
MEAN	19.8	18.1	2.1
SEM	$\pm$1.8	$\pm$3.1	$\pm$0.3

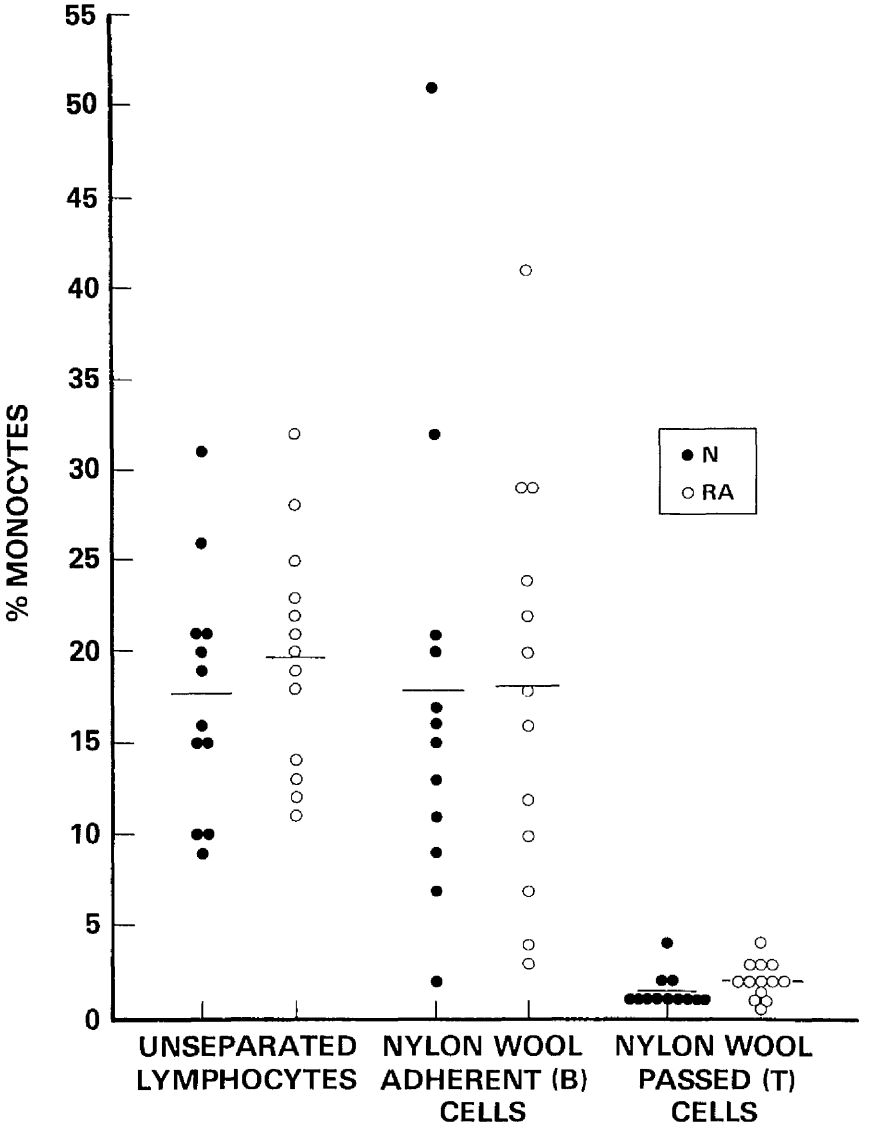


FIGURE 34 - MONOCYTE ENUMERATION IN RHEUMATOID AND NORMAL CELL SUSPENSIONS.

DISCUSSION.

Nonspecific esterase staining proved to be a simple method of enumerating mononuclear cells in various types of cell suspension. It was necessary to use the pararosanaline dye within a few hours of preparation otherwise staining was unsatisfactory. It was not possible to reuse pararosanaline dye (which had to be discarded after use) while methylgreen could be reused on multiple occasions. "Ghost" cells and polymorphs were not included in the counts, these two types of cell being easily distinguishable from lymphocytes and monocytes. The functional status and viability of lymphoid cells failing to take up methylgreen was unknown.

No difference[8] between rheumatoids and normals in the proportions of monocytes in any of the three cell suspensions were observed in this study. It was reasonable to conclude therefore that the radiation enhancement failure observed in rheumatoids was not due to a numerical deficiency of monocytes in rheumatoid cell suspensions. However, the functional status of monocytes in rheumatoid cell suspensions was not evaluated and could have been abnormal. It was anticipated that B cell preparations would be monocyte enriched in view of the adherence properties shared by these two cell populations. The fact that B cell preparations were not enriched suggests that a signifi-

cant number of monocytes were trapped on the nylon wool column. However, this loss was equivalent in both groups. Finally, passage of unseparated lymphocytes over nylon wool resulted in an almost monocyte-free T cell suspension.

SUMMARY.

The percentage of monocytes in the three principal cell suspensions used in previous studies [unseparated, nylon wool passed (T) nylon wool adherent (B)] were found equivalent between rheumatoids and normals. Unseparated and B lymphocyte suspensions contained larger numbers of monocytes than T cell suspensions, which contained very few. In particular it was evident that monocytes were not differentially depleted in the preparation of rheumatoid cell suspensions. If indeed only a small percentage of monocytes were required for the PFC response, then monocytes were present in more than adequate numbers. While no difference in the proportion of monocytes was demonstrated nothing could be concluded regarding the functional status of these monocytes. It was evident that failure of radiation enhancement was not due to numerical deficiency of monocytes.

CHAPTER 7

ENUMERATION OF Ia^+ T LYMPHOCYTES.

(STUDY 9)

- INTRODUCTION
- PATIENTS AND METHODS
- RESULTS
- DISCUSSION
- SUMMARY

Fortis dura coquit

(The brave man digests hard things)

Richelieu 1585-1642.

INTRODUCTION.

Realization of the potential importance of Ia^+ cells emerged from observations of the genetic control of immune responses in rodents. McDevitt and Benacerraff[305] demonstrated single gene control of the immune response in guinea pigs to the linear amino acid homopolymer poly-L-lysine. Subsequent work[306, 307] resulted in identification of the I region. Furthermore, it was recognized that there were subregions which had differing effects on responses to specific antigens. In particular the I-J subregion was noted to have suppressor activity.

Using specific alloantisera a number of I region gene products (Ia antigens) have been defined in the mouse and guinea pig. These antigens are expressed on B lymphocytes but also on monocytes and some subpopulations of T cells[308]. Ia antigens on T cells are serologically distinct from those Ia products on B cells. Products of the I-J and I-C subregions have been found associated with suppressor T cells and soluble suppressor factors, while other products of the I-J subregion and I-A subregion have been associated with helper T cells and soluble helper factors. While the precise relationship between Ia antigens and immune response (Ir-1) genes has not been defined, it is clear that these antigens play a role in immune response

mechanisms, since anti-Ia sera inhibit various in vitro assays of lymphocyte function, absorb various soluble T cell factors and eliminate certain specific functional subpopulations of T and B lymphocytes and monocytes.

I region gene products play an important role in the mixed lymphocyte reaction[309] (MLR) and graft versus host reaction[310] (GVHR). In both reactions it is thought that I-A and I-E sub-region products provide the principle stimulus which induces the development of cytotoxic T lymphocytes.

I region genes have been noted to have an effect not only on the immune response of individual cell types but also on the interactions which occur between different cell types. In respect of the former it has been noted Benacerraf and Germain [307] that:

(a) Control of specific immune responses by Ir genes is restricted to T dependent antigens, there being no record of control over T independent antigens.
(b) In cases of Ir gene control of antibody responses, the stimulation of specific helper T cells appears to be a critical step determined by these genes.
(c) Furthermore, it is postulated that the development of B cell memory is under Ir gene control, since it is dependent on helper T cell function.

(d) It has been observed that in selected (rodent) systems, Ir genes have a definite and reproducible effect on the type of antibody produced[311, 312], although the mechanism is unclear.

In respect of the latter[307]:

(a) In the last several years it has been shown in guinea pigs[313] and mice[314, 315] that I region genes control antigen presentation by macrophages play an important role in this phenomenon. Furthermore, macrophage Ia antigens contribute to the specificity of T cell clones stimulated.

(b) Experiments in mice[316] have indicated that Ir genes play a critical role in T cell- B cell interactions as well as monocyte- T cell interactions.

It can be seen from the foregoing evidence, that I region genes can determine a variety of recognitive and regulatory functions of the immune response. The mechanism of recognition is not clear, but may depend upon two cell receptors, one recognizing a specific antigen and the other an I region product, or on a single receptor capable of recognizing both. The nature of the receptor is unknown, although immuno-precipitation techniques have discovered two polypeptides of 29,000 and 34,000 daltons which together define components of Ia antigens. The

D locus genes of the human MHC have been considered functionally equivalent to the I region genes in the mouse. Investigation has been more difficult in humans, and although there has been substantial progress made, understanding is still incomplete.

Human Ia antigens were initially recognized with multiple pregnancy sera as a series of HLA linked alloantigens primarily represented on B lymphocytes[317]. Later they were detected by hetero-antisera raised against isolated protein from cell membranes[318]. Not confined to B cells, Ia antigens have been recognized on monocytes, some T cells, precursors of myeloid and erythroid cell series, leukemic blast cells and epidermal Langhans cells. A recent study[319] examining Ia bearing T lymphocytes in normal humans using absorbed rhodamine conjugated rabbit anti Ia antiserum, found that 1.7 - 8% of purified (99.5%) peripheral blood T cells were Ia positive. When T cells were activated by culture in conditioned medium, 96% of cells subsequently expressed Ia antigens, suggesting either that Ia positivity was induced or that it was acquired from the conditioned medium. The Ia^+ T lymphocyte population was shown to contain cells responsible for the generation of allogeneic helper activity, since elimination of Ia^+ cells using anti Ia antiserum resulted in loss of help to an allogeneic B cell preparation. Additional

evidence[320], using different methodology, suggested that more than one subset of T lymphocytes could be activated to express Ia antigens, and that this phenomenon was due to de novo expression of Ia antigens on Ia^- T cells, rather than a consequence of clonal expansion of a small subset of Ia^+ cells. Furthermore, T cells which failed to proliferate on exposure to soluble antigen did not express Ia like molecules. Not all authors, however, have recognized Ia^+ cells amongst resting T cell populations[320].

Kunkel's group[321] have carried out enumeration experiments in normal subjects and in patients with rheumatoid arthritis. Using a direct fluorescence assay employing $F(ab')_2$ fragments of rabbit anti-Ia antibodies, a normal range of 0-6% Ia^+ T cells was defined for the normal population. Almost all rheumatoids exceeded the upper range of normal and varied from approximately 3-35%. Somewhat higher values were obtained using the same anti-Ia rabbit antiserum by a bovine RBC rosette assay. It was observed that patients with a normal number of Ia^+ cells were clinically inactive. In these studies the Tγ fraction of normal and rheumatoid subjects was found to be enriched (up to 50%) with Ia^+ cells, although they were also present in substantial numbers in the non-Tγ fraction in both groups. (The percentage of Ia^+ cells exceeded the percentage of Tγ cells in some patients). It

was of note, that the small percentages of Ia^+ T cells found in normal subjects and diseased individuals were primarily small lymphocytes, whereas the high percentages of Ia^+ T cells found following in vitro stimulation were almost entirely due to large blastoid cells.

The exact origin and significance of Ia+ T cells remains to be elucidated. The observations that Ia^+ T cells are increased in several active disease states (rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease) and that a marked increase follows the administration of tetanus toxoid or PPD suggest that these cells might be recruited into the circulation following antigenic stimulation. Since it is not known how selective a fraction the Tγ fraction really is, it is premature to draw any conclusion from the observation that Ia^+ cells are more common in the Tγ fraction[321, 322], although not confined to this fraction. While Ia positivity in macrophages has been considered a marker for inducer (helper) activity in mouse/listeria monocytogenes model, evidence for similar significance in the human T cell is less compelling. Since Ia positivity has been associated with cells having both helper and suppressor activity, it is possible that the level of Ia^+ cells in peripheral blood is merely a nonspecific index of immune stimulation and does not identify a single functional subset of mononuclear cells.

The proportions of Ia^+ cells in nylon wool passed (T lymphocyte) suspensions from rheumatoids and normal subjects were enumerated in London, in order to define any differences between the two groups. The study was designed to be descriptive and no attempt was made to examine the Ia^+ cell content of the Tγ and T nonγ fractions. Since the significance of Ia positivity is ill defined, it was realized prior to the commencement of the study, that should differences be discovered between the two groups, there would be difficulty in ascribing to them their true importance. The objectives of this study were (a) to enumerate the numbers of Ia^+ T cells in rheumatoids and normals and (b) to confirm the findings of Kunkel's group using a different technique.

PATIENTS AND METHODS.

Six patients with classical rheumatoid arthritis (CF, RR, MS, NI, TM, SW) and six normal controls (mf, jd, db, jo, ke, nb) were chosen for study (Appendices 1 and 2). T lymphocytes were separated from 30 mls of fresh heparanized blood by the Ficoll-Hypaque centrifugation and nylon wool separation methods previously described in Chapter 3. Following washing three times in RPMI 1640, non-adherent (T) lymphocytes were enumerated and then resuspended in RPMI 1640 to a concentration of $5x10^5$ cells per ml (500 cells per microlitre). Enumeration was conducted on HLA typing trays under paraffin oil (in order to prevent drying of the preparation). For each subject, one drop of mineral oil was pipetted into each of six wells. One microlitre of a 1 in 100 dilution of monoclonal anti-Ia antibody (New England Nuclear) was introduced by a Hamilton micropipette into each of four wells, while 1 microlitre of RPMI 1640 was added to each of the two remaining wells. Then, 1 microlitre of T cell suspension (500 cells per microlitre) was pipetted into each well, care being taken, as with the initial preparation to introduce the pipette under the layer of paraffin oil prior to ejection. The typing tray was shaken gently by rubbing it over the under surface of a hemogglutination tray in order to mix lymphocytes and antiserum. The preparation was then incubated at room temperature for forty-five minutes, following which 5 microlitres of rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario) were introduced

by micropipette to each well and the tray shaken gently to mix complement, antiserum and lymphocytes. Thereafter the preparation was incubated for one hour at room temperature, following which 1 drop of eosin was added to each well to stain the preparation. After 5 minutes, one drop of 37% Formaldehyde was added to each well to fix the cells. A microscope cover slip was then placed over the top of all wells and the bottom of the tray flooded with paraffin oil to prevent drying of the preparation. The preparations were enumerated under an inverted microscope, at least 100 cells being counted in order to determine the per cent kill of T lymphocytes in this cytotoxicity assay. A killed cell (Ia^+) was recognized by its dark colouration while viable (Ia^-) cells were pale in colour. The percentage kill was determined in this fashion for each of the wells containing anti-Ia antiserum as well as the two control wells containing only lymphocytes and RPMI. Values from control wells were subtracted from test wells to give the actual (control corrected) anti-Ia antiserum-dependent percent kill.

RESULTS.

Under the inverted microscope killed and non-killed lymphocytes were scattered about the bottom of each well. In every case the percent kill in wells containing anti-Ia antiserum exceeded that in control wells. When corrected for control kill (RPMI wells) a range of Ia^+ T cells varying from 0.8 to 3.25%, with a mean of 1.74% was observed in normal subjects (Table 27). With one exception, normal subjects were below 2.25%. The range of percent Ia^+ T cells in rheumatoids varied from 2.25 to 9% with a mean of 4.14%. No rheumatoids were enumerated as being below 2.25%. The most active rheumatoid (M.S.) had the highest percent of Ia^+ T cells (9%). Thus, while there was some overlap in the ranges of the two groups, 50% of rheumatoids studied were above the upper limit of the range of normal in controls. (Figure 35).

DISCUSSION.

This study indicated that a range of values exists for both normals and rheumatoids. The small but significant (and variable) cytotoxicity in control wells, illustrated the importance of such controls. Although the magnitude of the values differed from those reported by other authors[17] (possibly reflecting a difference in techniques), the observation of a

TABLE 27 - ENUMERATION OF Ia^+ T CELLS IN R.A. AND NORMAL.

NORMAL

Subject	$\%Ia^+$	Control	Control Corrected $\%Ia^+$
m.f.	2.75	1	1.75
j.d.	4	0.75	3.25
d.b.	2.7	0.5	2.2
j.o.	3.7	1.5	2.2
k.e.	1.5	1.25	0.25
n.b.	1.3	0.5	0.8
MEAN	2.66	0.92	1.74

R.A.

Subject	$\%Ia^+$	Control	Control Corrected $\%Ia^+$
C.F.	5.5	1.5	4
R.R.	14.8	10.5	4.3
M.S.	11	2	9
N.I.	3.8	1	2.8
T.M.	4	1.5	2.5
S.W.	3	0.75	2.25
MEAN	7.02	2.88	4.14

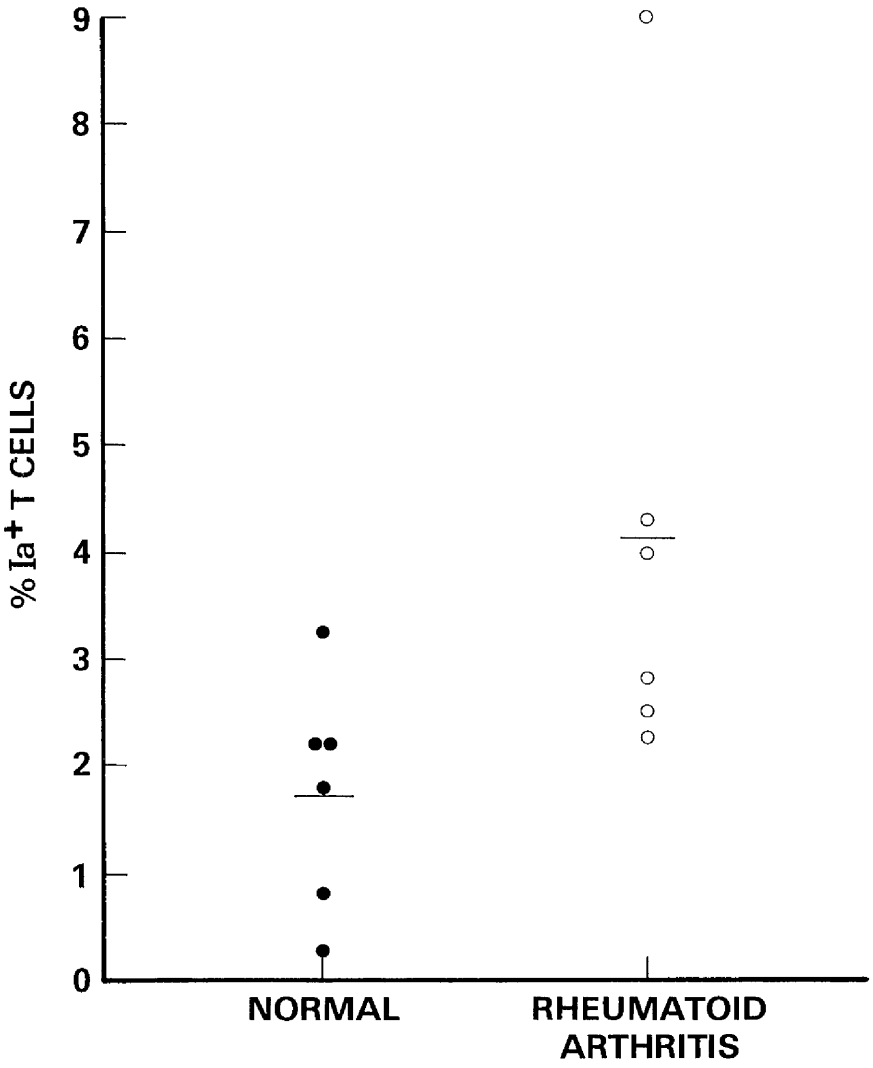


FIGURE 35.- ENUMERATION OF Ia^+ T CELLS IN NORMAL AND RHEUMATOID NYLON WOOL PASSED CELL SUSPENSIONS.

high number of Ia^+ T cells in rheumatoids was confirmed. Furthermore, as has been previously reported[17], the most active patient had the highest number of Ia^+ T cells. However, these results cannot be regarded as wholly satisfactory, since the number of subjects evaluated was small, contamination with B cells and monocytes (also Ia^+) although small was not defined, and the single active patient cannot be regarded as a trend. Nevertheless, the technique was standardized in both rheumatoid and normal control groups and therefore the results were at least consistent with previous reports. Serial study of a large number of rheumatoid and control subjects would be needed to better define the range of Ia^+ T cells in the two groups, and to demonstrate any relationship between disease activity in rheumatoids or intercurrent illness in controls and the degree of Ia positivity.

SUMMARY.

D locus genes in humans are thought to play an equivalent immunoregulatory role to I region genes in mice. Cells bearing Ia antigens can be recognized using various anti-Ia antisera. B cells, monocytes and some T cells may bear such Ia antigens. The significance of Ia positivity on T cells has not been defined. It is evident, that Ia positivity may be expressed on previously Ia^- T cells, that both the Tγ and T nonγ fractions contain Ia^+

cells and that in several diseased states, there is an increase in the number of Ia^+ T cells particularly when the disease is active. Furthermore, Ia^+ monocytes are believed to play a role in the inductive phase of the immune response (see Chapter 6). In this study the mean percentage of Ia^+ cells in nylon wool passed suspensions was 4.14% in rheumatoids and 1.74% in normal controls. The highest number (9%) of Ia^+ T cells was recorded in a patient with active disease. While the numbers of Ia^+ T cells were less than reported by other workers (possibly due to a difference in techniques), the increased numbers in rheumatoids (particularly with active disease) were consistent with other reports. Serial studies using larger numbers of subjects are required to examine the relationship between disease activity and the degree of Ia positivity.

CHAPTER 8

ENUMERATION OF T CELL SUBPOPULATIONS BY MONOCLONAL ANTIBODIES

(STUDY 10)

- INTRODUCTION
- PATIENTS AND METHODS
- RESULTS
- DISCUSSION
- SUMMARY

"And in the end
Are more beginnings than men
Shall name or know today."

Edwin Arlington Robinson
Merlin.

INTRODUCTION.

It can be appreciated from the foregoing chapters, that development of new methodologies allowed first the recognition of lymphocytes amongst the total leukocyte population, then the differentiation of B and T lymphocytes, and more recently the definition of T lymphocyte subpopulations. It is evident that various techniques have limitations and this is particularly so in the identification of lymphocyte subsets. It appears that (a) T cell Fc receptor expression may be unstable and transform from Tγ to Tμ[323], that (b) Tμ cells in certain circumstances may have a net suppressor activity[324], and (c) that some Tγ cells may not be T cells at all[325]. Having levelled these criticisms, it is also true, that the techniques described so far in this thesis, were capable of identifying differences in immunoregulation between patients with rheumatoid arthritis and normal controls. However, the appearance of a new methodology which accurately identified T cell subsets showing some degree of functional homogeneity and consistency, offered substantial advantage in the study of helper/suppressor modulation of the immune response.

Hybridoma technology has developed over the last ten years to meet requirements for large quantities of various defined types of specific antibodies with uniform avidities. These

monoclonal antibodies have been produced by various companies, each competing on the basis of producing antibodies with superior performance (predictability, selectivity and characterisation). The hybridoma technique involves the fusion of antibody producing cells harvested from the spleens of immunized mice, with a mouse or rat myeloma cell, which lacks the enzyme hypoxanthine phosphoribosyl transferase. This metabolic defect allows elimination of infused cells, by the addition of hypoxanthine, aminopterin and thymidine to the cultures, after fusion. Cells which have fused and proliferate are cultured at limiting dilutions in order to allow isolation of individual clones. Products of these clones are characterised and some cells thereafter selected for perpetuation. Thus, monoclonal antibodies are produced by cells descended from a single hybridoma cell and have specificity for the antigen used in the initial mouse immunization.

Permanent cell lines of myeloma cells may be obtained commercially or induced in the laboratory[326]. Specificity of the monoclonal antibody produced is dictated by the antigen used in immunizing the mouse to generate antibody secreting cells. Historically the realization that certain animal viruses induced

the formation of multinucleated cells by fusing together single cells, provided a method by which Myeloma and antibody secreting cells could be fused[327].

Successful fusion between immunoglobulin producing wells and non-immunoglobulin producing cells was achieved relatively early on[328], while fusion between two types of immunoglobulin producing cell has been more recent[329, 330, 331]. It has been demonstrated that the new immunoglobulin molecules produced are the result of mixed associations between heavy and light chains from the two parent cells[231], a process which occurs intracellularly. Thus in hybrid cells the expression of one isotype and idiotype does not exclude the expression of another. Furthermore, it is possible to isolate hybrid lines producing different antibodies directed against the same antigen.

Of relevance to the studies being reported, monoclonal antibodies to T cell subsets have been commercially produced by hybridoma techniques, using human T cells as the antigen used in mouse immunization. Various companies have designated their antibodies differently. Orthopharmaceuticals designate their products OKT3, OKT4, OKT5, OKT6 and OKT8, while Becton Dickinson market Leu 1, Leu 2 and Leu 3. Orthopharmaceutical products OKT4 (monoclonal antibody directed against T helper cells) and OKT5

(monoclonal antibody directed against T suppressor cells) were used in the study to be later reported, since the product appeared to have superior performance characteristics.

Several diagnostic and therapeutic applications for monoclonal antibodies have been suggested.

Diagnostic:

1. Monitoring the efficacy and safety level of immunoregulatory compounds in Phase I and Phase II trials. This may result in reduced drug dosages and improve therapeutic decision making.
2. Immune profile tests. Since abnormal proportions of T cell subsets have been observed in several disease states a panel of monoclonal antibodies may be useful in disease differentiation.
3. Leukemia typing. Definition of subgroups and differentiation of several leukemic states may be facilitated by this methodology. This may result in earlier diagnosis and the defining of more effective forms of therapy for individual subsets.

Therapeutic:

1. Prevention of graft or transplant rejection. Used as an antilymphocyte agent, monoclonal antibodies may offer advantage in suppressing the immune response and preventing graft rejection.

2. Leukemia therapy. Specific therapy directed against leukemic cells or T cell lymphomas offers significant advantage over the relatively non-specific forms of therapy now available. However, any therapeutic use for monoclonal antibodies depends on their being safe, as well as effective.

Apart from clinical considerations, these antibodies have been used to examine the ontogeny of T lymphocytes. As previously described the earliest lymphocyte precursors lack mature T cell antigens on entering the thymus[332]. This 10% of thymic cells are reactive with monoclonal antibodies anti-T9 and anti-T10. With maturation[333], thymocytes lose T9, retain T10 and acquire T4, T5 and T6. With further maturation T6 is lost and T1 and T3 acquired. Finally in the mature thymocyte segregation occurs[334], either T4 or T5 being lost and two separate populations being identifiable; those which are T10 +, T1+, T3+, T4+ and those which are T10+, T1+, T3+ and T5+. Next, it appears that T10 is lost, since all peripheral T cells are T1+, T3+ and T10-. The T4 antigen is expressed on 55 to 65 per cent and T5 antigen on 20 to 30 per cent of peripheral blood T cells[335, 336]. Cells bearing the combination of T1+, T3+, T4+ are considered helper T cells, while those bearing the combination of T1+, T3+, and T5+ are considered suppressor T cells. Unlike maturing thymocytes T4 and T5 antigens are expressed on mutually exclusive subsets of mature T cells.

The activities of T4+ and T5+ subsets have been defined in a number of ways. The proliferative capacity of the B cell population to soluble antigens is contained within the T4 subset, whilst the response to cell surface antigens in mixed lymphocyte cultures involves both T4+ and T5+ populations. The T4+ cell response to phytohemagglutanin is greater than that of T5+ cells, both populations responding comparably to Conconavalin A. The cytotoxic killer cell belongs to the T5+ population, not being recognised in the T4+ subset. T4+ cells have been shown to have helper/inducer activity in T-T, T-B and T-macrophage interactions. Thus T4+ cells can augment the cytotoxicity of T5+ effector cells without themselves being cytotoxic. They can induce B cells to proliferate and differentiate into immunoglobulin containing plasma cells[337, 338]. Only the T4+ subset has been recognized as producing lymphocyte mitogenic factor (LMF), which induces the proliferation of all major lymphocyte subclasses, and T helper factors which initiate B cell immunoglobulin synthesis[338]. It is apparent that T4+ cells regulate the immune response by cell-cell interaction as well as by the release of soluble regulatory molecules. The T5+ subset contains cells which after activation with Conconavalin A suppress the autologous proliferative response to allo-antigens[230, 336], and can suppress B cell immunoglobulin production[339]. T4+ and T5+ subpopulations are programmed for

their respective helper and suppressor function independently of their ability to recognize and react to nonspecific polyclonal mitogens and antigens, since both subpopulations respond equally well to Concanavalin A but only the T5+ population becomes suppressive.

Recently the hybridoma antibody OKMI, has identified two distinct populations of monocytes: an adherent population of large cells bearing Ia^+ determinants and a non-adherent population of small Ia^- cells[340]. OKMI+ Ia^- cells were found to be contaminating most fractionated mononuclear cell subsets including the null cell population. In a recent comparison of enumeration techniques[325], Reinherz et al drew attention to the lack of correlation between T cell subsets defined by monoclonal antibodies and those defined by Fc receptors. The Tμ subpopulation was shown to contain both helper (OKT4+) and cytotoxic/suppressor (OKT5+) populations and was similar to the unfractionated T cell population. The Tγ subpopulation contained few OKT3+ cells (monoclonal antibody identifying all T cells) or subsets defined by OKT4 or OKT5 monoclonal antibodies. Rather, the Tγ population was comprised largely of Ia^- cells possessing OKMI monocyte antigen. Furthermore, OKT4+ and OKT5+ T cell subsets contained few Tγ cells but were comprised mainly of Tμ cells. Since Tγ depletion has been shown in a previous chapter to result in enhanced immunoglobulin synthesis from B cells, the absence of identifiable

suppressor cells in this subpopulation remains to be resolved. However, these observations are consistent with the notion that not all Tγ cells suppress and not all suppressor cells are Tγ cells.

Prior to the development of the OKT series monoclonal antibodies, anti-TH_2 antibodies were used to study T cell subpopulations in a number of disease states[341, 342]. (TH_2+ corresponds to OKT5 and TH_2^- to OKT4 in their frequency of occurrence and functional characterisation of T cells). TH_2+ (suppressor) cells were observed to be reduced in acute graft versus host disease (GHVD) and were variable (increased in 4 and decreased in 2 patients) in chronic GVHD) Since I^aantigens were found on TH_2+ cells in two of the four patients with increased numbers of TH_2+ cells, the concept of Ia positivity as a marker of activity, rather than of a particular functional subset was strengthened. Decreased numbers of TH_2+ cells in a patient with auto-immune disease and increased numbers in a patient with agammaglobulinaemia, suggested that definable alterations in functional subsets could be related to clinical entities. The degree of lymphocyte activation was characterised by a predominence of TH_2^- Ia+ cells in the blood of the first patient and a predominence of TH_2+ Ia+ cells in the second patient.

OKT3, T4 and T5 monoclonal antibodies have been utilised in the study of patients with multiple sclerosis[343]. A reduction of T5+ cells was noted in 73% of patients with active multiple sclerosis but only 5% of patients with inactive disease. In 5 patients serial analyses demonstrated a correlation between the absence of the T5+ subset and disease activity, suggesting that immunoregulatory abnormalities contributed to the pathogenesis of the disease. Recently Ceuppens and Goodwin[344] have enumerated T cell subsets in rheumatoid arthritis and normal subjects using monoclonal antibodies. Ia+ T cells were found in 8 $\pm$ 6% of normals and 17 $\pm$ 13% of rheumatoids. In both groups they were found on OKMI+, OKT3- and OKMI+ OKT3- cells. A substantial number of E-rosetting (T) cells, stained with OKMI antibody in both groups casting doubt on the specificity of this monoclonal antibody for the granulocyte monocyte series of cells. OKT8+ (suppressor/cytotoxic) cells were decreased in rheumatoids (28 $\pm$ 3%) compared with normals (36 $\pm$ 2%).

In the study which follows OKT4 (anti helper/inducer cell) and OKT5 (anti suppressor/cytotoxic cell) monoclonal antibodies were used in a microcytotoxicity assay to enumerate T cell populations in rheumatoid and normal, nylon wool passed cell suspensions.

PATIENTS AND METHODS.

15 patients with classical or definite rheumatoid arthritis (JG, DM$_1$, MK, FM, DK, PL, CS, JL, SN, CK, EM, AT, RM, SW, TM) and 7 controls (ds$_1$, ac, mf, cm, db, ke, ce) were selected for study (Appendices 1 and 2). T lymphocytes (nonadherent mononuclear cells) were separated from 30 mls of fresh heparanized blood by the method of density gradient centrifugation and nylon wool separation previously described. T cells were finally suspended to a concentration of 500 cells per microlitre in RPMI 1640 containing 5% sheep red blood cell absorbed fetal calf serum. Single batches of OKT4 and OKT5 monoclonal antibodies (Ortho-pharmaceutical Corporation) were aliquoted andfrozen. Repeated thawing and refreezing were reduced to a minimum in order to preserve antibody potency. Several dilutions of monoclonal antibodies were tested but a 1 in 50 dilution of 1 microgram/microlitre OKT4 and a 1 in 10 dilution of 1 microgram/microlitre OKT5 were found to be optimal. Enumeration by a microcytotoxicity assay was conducted on HLA typing trays, which were kept on ice during preparation. One drop of paraffin oil was pipetted into each well to be used, in order to prevent drying of the subsequent preparation. Then 1 microlitre of OKT4 monoclonal antibody or 1 microlitre of OKT5 monoclonal antibody at appropriate concentration (see Appendix4) was introduced into each of several test wells with a Hamilton micro-pipette. One microlitre of RPMI 1640 was introduced into

each of several control wells. Care was taken in introducing reagents to ensure that they were pipetted under the paraffin oil. To each of the test and control wells was added 1 microlitre of T cell suspension (500 cells per microlitre), care again being taken to ensure that cells were pipetted under the paraffin oil. The typing tray was then shaken gently by rubbing it over the undersurface of a hemagglutination tray in order to mix cells and antibody. The preparation was maintained on ice for 30 minutes, and was agitated in the above fashion for 30 seconds every 10 minutes. At the end of this time, 5 microlitres of rabbit complement (Cedarlane Laboratories, Hornby, Ontario) were added to each well in the aforementioned fashion. The tray was agitated initially and then for 30 seconds every 10 minutes, incubation being conducted at 37°C for a total of 30 minutes. At the end of this time, one drop of eosin was added to each well and after 2 minutes one drop of 37% formaldehyde. This procedure stained and fixed the cell suspension. Finally a microscope cover slip was placed over the top of all wells and the bottom of the typing tray flooded with paraffin oil to prevent drying of the preparation. Introduction of paraffin oil required considerable care in order not to introduce air bubbles, particularly in the region of the typing well. It was important not to flood the top surface of the coverslip as this resulted in difficulty visualizing the cells by microscope. Trays

were read under an inverted microscope. At least 100 cells were counted in several different areas of each well and the average percent kill (cytotoxicity) determined in replicate. Killed cells were recognized by their dark appearance, whilst healthy cells were bright and clear. Counting several areas of the well was important since (a) killing was not uniform and (b) the convex shape of the bottom of the typing well tended to result in a higher density of cells at the edge. The cell concentration of 500 cells per microlitre was optimal for accurately estimating cytotoxicity since a greater number of cells resulted in piling up of cells at the edge of the well and a cell density which prohibited accurate manual counting.

The number of OKT5+ cells was compared to the number of Tγ cells previously determined. However, in two individuals OKT5+ and Tγ+ cells were enumerated simultaneously before and after Tγ depletion.

RESULTS.

Original data are displayed in Appendix 4. Wide ranges of T helper (OKT4+) and T suppressor (OKT5+) cells were identified in rheumatoid subjects (Table 28), but there was no significant difference between the numbers of OKT4+ cells in R.A. (mean 46%, range 27-65%) and normals (mean 42%, range 31-50%), or between numbers of OKT5+ cells in R.A. (mean 15%, range 3-22%) and normals (mean 16%, range 9-21%), at the OKT4 concentration of 1 in 50 and OKT5 of 1 in 10 (Figure 36). Serial dilutions were not conducted on rheumatoids since dilution experiments conducted in normals indicated the above dilutions as being optimal to observe cytotoxicity, i.e. lower dilutions did not produce significantly greater cytotoxicity while in higher dilutions cytotoxicity declined. (Table 29) No direct relationship was evident between the percentage of Tγ cells (previously determined) and the percentage of OKT5+ cells in either rheumatoids or normals (Table 30). It was observed in both groups that the percentage OKT5+ cells could be either more or less than the percentage of Tγ cells. Overall OKT5+ and Tγ+ mean percentages in normal subjects were equivalent (11.6% and 11.5%), while in rheumatoids the mean percentages of OKT5+ cells (16.1%) exceeded that of Tγ+ cells (10.5%). Not all patients were enumerated by both methods and therefore not all patients from Table 28 are represented.

TABLE 28 - ENUMERATION OF OKT4+, OKT5+ CELLS IN R.A. AND CONTROLS

	%CYTOTOXICITY (CONTROL CORRECTED)				
	R.A.			NORMAL	
	OKT4 (1:50)	OKT5 (1:10)		OKT4 (1:50)	OKT5 (1:10)
JG	65	21	da_1	50	19
DM_1	49	19	ac	31	20
MK	36	19	mf	50	9
FM	44	22	cm	44	16
DK	45	7	db	36	16
DL	55	11	ke	40	21
CS	45	22	ce	43	10
JL	40	21	MEAN	42	16
SN	52	17			
CK	60	10			
EM	45	8			
AD	30	19			
RM	51	13			
SW	39	6			
TM	27	3			
MEAN	46	15			

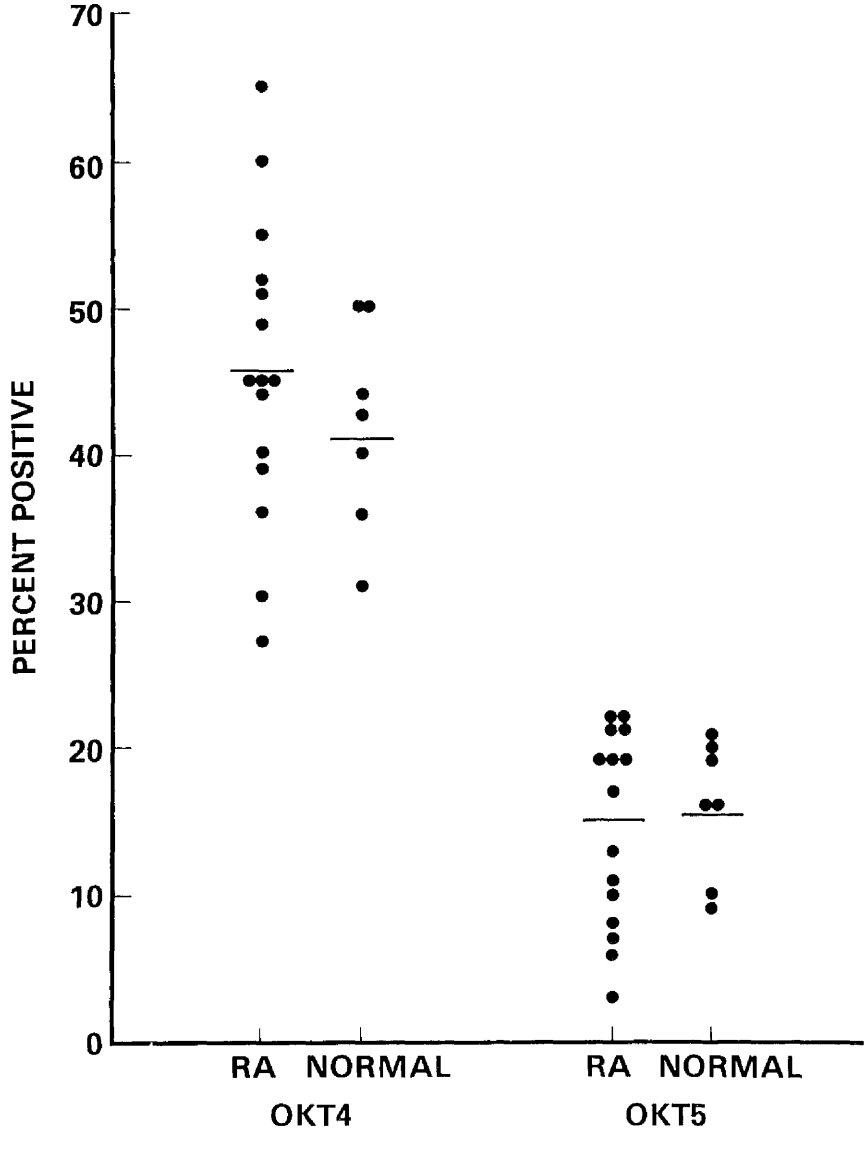


FIGURE 36 - ENUMERATION OF OKT4+ AND OKT5+ T CELLS IN R.A. AND NORMAL SUBJECTS.

TABLE 39 - EFFECT OF DILUTION OF MONOCLONAL ANTIBODIES OKT4 AND OKT5 ON CYTOTOXICITY.

% CYTOTOXICITY (CONTROL CORRECTED)

	OKT4				OKT5			
	1/5	1/10	1/50	1/100	1/5	1/10	1/50	1/100
ds_1	42	38	50	21	22	19	16	9
ac	46	42	31	7	12	20	10	4
mf	-	-	50	-	-	9	-	-
cm	-	-	44	-	-	16	22	-
db	-	-	36	-	-	16	15	-
ke	-	-	40	-	-	21	-	-
ce	-	-	43	-	-	10	7	-
MEAN	44	40	42	14	17	16	14	7

TABLE 30 - RELATIONSHIP BETWEEN OKT5+ AND Tγ CELLS.

	R.A.			NORMAL	
	%Tγ	OKT5		%Tγ	OKT5
JG	8	21	mf	6	9
DM_1	7	19	cm	13	16
MK	10	19	db	16	16
FM	15	22	ke	11	6
DK	15	7	ce	12	10
DL	9	11	Mean	11.6	11.4
CS	11	22			
JL	17	21			
SN	6	17			
CK	5	10			
EM	13	8			
Mean	10.5	16.1			

In a single experiment where T cells were enumerated with monoclonal antibodies before and after total Tγ depletion by the chicken rosette method previously described, equivalent percentages of Tγ cells (12% and 11%), OKT5+ (10% and 6%) and OKT4+ (43 and 37%) cells were present in two normal subjects prior to depletion. (Table 31). The effectiveness of subsequent Tγ depletion was confirmed by a total failure of rosette formation on rerosetting (0% in both subjects). Following depletion the proportion of OKT5 cells dropped significantly (10% to 0.5% in one subject and 6% to 2% in the other. Simultaneous with this, the proportion of OKT4+ cells increased from 43 to 47% in one subject and from 37 to 47% in the other.

The ratio of OKT4+ to OKT5+ cells was used to reflect the help/suppression balance present in each individual (Table 32). In rheumatoids the mean ratio was 4:1 (range 1.6 to 9:1) while in controls it was lower at 3.3:1 (range 1.9 to 5.6:1).

Although these differences were relatively small and there was considerable overlap, review of clinically active patients (Figure 37) demonstrated that the T4/T5 ratio (or helper/suppressor balance) was higher in these patients (mean 6:1, range 3-9:1), than in relatively inactive rheumatoids (mean 2.8, range 1.6-6:1) or normals (mean 3.3:1, range 1.9-5.6:1). The difference in ratio was accounted for principally by a reduction in the number of suppressor cells (mean 9%, range 3-21%) in active patients

TABLE 31 - EFFECT OF Tγ DEPLETION ON OKT5 POSITIVITY.

		c.e.	k.e.
	% Tγ	12%	11%
Pre Tγ depletion	% OKT5+	10	6
	% OKT4+	43	37
Post Tγ depletion	% Tγ	0%	0%
	% OKT5+	0.5	2
	% OKT4+	47	47

TABLE 32 - HELP/SUPPRESSION BALANCE IN R.A. AND NORMAL.

OKT4/OKT5 RATIO

R.A.		NORMAL	
JG*	3:1	ds_1	2.6:1
DM_1	2.6:1	ac	3.1:1
MK	1.9:1	mf	5.6:1
FM	2:1	cm	2.8:1
DK*	6.4:1	db	2.3:1
PL*	5:1	ke	1.9:1
CS	2:1	ce	4.3:1
JL	2:1		
SM	3:1		
CK	6:1		
EM*	5.6:1		
AT	1.6:1		
RM	3.9:1		
SW*	6.5:1		
TM*	9:1		
Mean Ratio	4:1		3.3:1

* = Active

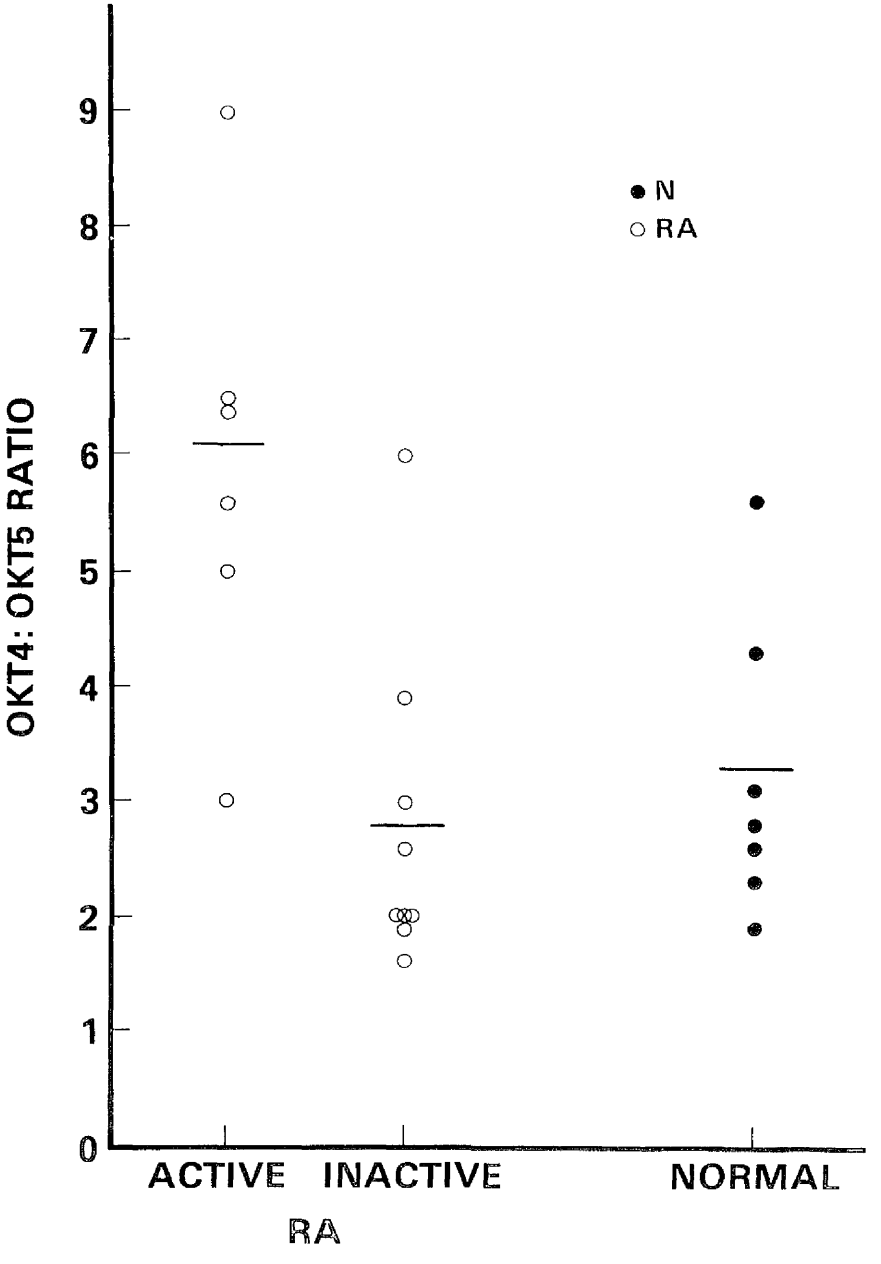


FIGURE 37 - COMPARISON OF HELP SUPPRESSION RATIO IN ACTIVE R.A., INACTIVE R.A. AND NORMAL CONTROLS

rather than an increase in OKT4 cells (mean 48%, range 27-65%), i.e. compared with overall group means of 15% (OKT5) and 48% (OKT4).

DISCUSSION:

In this study a microcytotoxicity assay using monoclonal antibodies OKT4 and OKT5 was shown to be a useful method of enumerating helper/inducer and suppressor/cytotoxic T cells. The ranges of OKT4+ and OKT5+ cells were similar in both groups, suggesting that between group differences in radiation enhancement, were not accounted for purely on the basis of an excess or deficiency of one particular subset of lymphocytes. Multiple area counting and replicate determinations allowed confidence to be placed in the accuracy of these results. It was clear from the maldistribution of killing and of cells that this approach be mandatory in enumerating T cells using this technique. The ranges of normal for OKT4+ and OKT5+ cells were slightly less than those reported by Reinherz and Schlossman and likely represented differences in monoclonal antibodies and techniques. However, an approximately 2:1 rationof OKT4+: OKT5+ means was in accordance with their observations.

One of the problems of group analysis is that heterogeneity within the group may go unrecognized. In addition, it is evident that in this system the ratio between OKT4+ and OKT5+ cells likely

determines the net effect of the help/suppression balance. Thus, when the data were analysed with respect to clinical activity, it was evident that patients with active disease had higher T4/T5 ratios, than those with inactive disease who approximated closely to the normal. In this subset of rheumatoids the shift in help/suppression balance towards help, was due to a relative decrease in the number of suppressor cells rather than an increase in helper cells. Whether the alteration in help/suppression balance (OKT4/OKT5 ratio) was primary or secondary to active disease could not be defined from the results of this study. However, serial enumerations conducted on the same subjects at times of differing disease activity, would allow description to be made of temporal fluctuations in the OKT4 to OKT5 ratio, and whether they occurred before or after the onset of clinically evident changes in disease activity.

The relationship between OKT5+ and Tγ cells was not completely defined. Historial evidence suggested that OKT5+ and Tγ cells should be one and the same i.e. suppressor T cells. While the specificity of OKT5 positivity has not yet been challenged, some controversy surrounds the Tγ cell. The failure to find a direct relationship between the number of OKT5+ and Tγ cells in the large number of enumerations conducted in this study, possibly reflected the fact that enumeration of OKT5+ cells was conducted on a different day from Tγ enumeration for each subject. It was noted that in normalsubjects, the number of Tγ cells was not constant but

varied between days -- a characteristic not defined for OKT5+ cells. The experiment conducted on two normal subjects (ce and ke) while limited by numbers, was possibly more reliable since enumeration of OKT5+ and Tγ cells was conducted simultaneously. Since the number of OKT5+ cells was markedly reduced by Tγ depletion, the results indicated that a large number of Tγ cells were also OKT5+. However, the number of OKT5+ cells did not reach zero in this experiment, further indicating the presence of OKT5+, Tγ- cells. Finally, it seemed reasonable to assume that there may also have been OKT5- Tγ cells in the preparation, although this could not be known for certain. Therefore it appeared that there existed a relationship between OKT5 positivity and Tγ positivity, and that if OKT5 was a marker for a suppressor T cell then it followed that Tγ was also a marker for such a cell. This reasoning was consistent with the observation that serial depletion of Tγ cells in normal subjects progressively enhanced the PFC response. Thus, it appeared that OKT5 and Tγ enumeration techniques examined in general ,different facets of the same cell, while each was capable of identifying some cells not identifiable by the other. Furthermore, since no association was shown in the earlier studies between disease activity and radiation enhancement failure, and in the absence of time series studies, it was concluded that the reduction in the number of suppressor T cells (i.e. alteration in help/suppression balance as reflected by OKT4/OKT5 ratio) in active patients, was dissociated from the effects of such cells on poly-

clonal immunoglobulin synthesis in vitro, as measured by the reverse hemolytic plaque forming cell assay.

While hybridoma techniques have provided new opportunities in immunology, the proliferation of this type of research is likely to be constrained by the relatively high cost of monoclonal antibodies. In these experiments, it was shown that a 1 in 50 dilution of OKT4 and 1 in 10 dilution of OKT5 monoclonal antibody were adequate to prdduce optimal cytotoxicity. That is to say, higher concentrations resulted in no additional killing in the majority of patients studied, and that lower concentrations resulted in a partial failure of cytotoxicity. Where reagents are in limited supply, there is an additional non-biological reason to define the optimal operating conditions in order to assess the feasibility of completing a given study. Present technology and the high cost of monoclonal antibody restrict functional studies on OKT4 and OKT5 depleted lymphocyte subpopulations. However, a relatively recent advance, the immunofluorescent cell sorter, promises to provide an efficient if not cheap method of obtaining cell suspensions of purified lymphocyte subpopulations.

This study reinforced the concept that simple numerical deficiencies in T cells did not in themselves account for difference observed between rheumatoids and normals. Although a disturbance in the help/suppression balance was described in

active rheumatoid arthritis, these data did not allow any conclusion to be drawn regarding their cause or effect relationship to that activity. Following the enumeration of monocytes, Tγ, OKT4+ and OKT5+ cells, it was evident that further progress could only be made by additional functional studies. Evidence from the first 10 studies suggested there was a functional defect in rheumatoid mononuclear cells (lymphocytes or monocytes), which was either intrinsic to one or several cell populations or impeded cell-cell communication (directly or indirectly).

SUMMARY:

In this study a recently developed technology was applied in the enumeration of T lymphocytes having helper and suppressor activity in patients with rheumatoid arthritis and normal subjects. No numerical differences were discovered between groups for either OKT4+ or OKT5+ subset. Although no direct association between OKT5+ and Tγ cells could be concluded, enumeration experiments were not conducted simultaneously on the same cell suspensions at the same time, thus any conclusion drawn from this comparison should be guarded. However, simultaneous experiments conducted on two normal subjects, suggested that Tγ and OKT5+ cells shared identity as evidenced by a marked reduction of OKT5+ cells when Tγ cells were completely depleted. It was evident, however, that not all Tγ cells were OKT5+ and therefore some were likely not suppressor cells. A disturbance in the help/suppression balance

was demonstrated in patients with active rheumatoid arthritis, when results were compared with those from patients with inactive disease or normal controls. While no cause or effect relationship was shown, it was noted that this shift in balance could be accounted for by a relative reduction in the proportion of suppressor cells. Simultaneous enumeration, time series studies and functional experiments with pure lymphocyte subsets (as defined by monoclonal antibodies), offer an opportunity to answer some of the remaining questions.

CHAPTER 9

PFC RESPONSE OF RHEUMATOID AND NORMAL LYMPHOCYTES IN AUTOLOGOUS AND ALLOGENEIC CULTURES (STUDY 11)

- INTRODUCTION
- PATIENTS AND METHODS
- RESULTS
- DISCUSSION
- SUMMARY

"Nature does nothing
without a purpose".

Aristotle 384-322 BC

INTRODUCTION.

In preceding chapters, indirect evidence has been cited to indicate that rheumatoid B lymphocytes have the capability to respond to helper T cell signals, i.e. the response from cocultures from B and non-irradiated T cells was greater than that from cultures of B cells alone. Thus it appears that R.A. B cells can respond although the extent of this response has not been defined. One possible explanation for the observed differences in radiation and Tγ depletion enhancement, between the two groups is that the B cell response in rheumatoids is limited, i.e. rheumatoid B cells can only respond up to a certain level. If this were true, then functional destruction of suppressor T cells (or their physical removal), or the provision of more helper signals by adding normal T cells, soluble helper factors or increased numbers of R.A. T-cells should not result in augmentation of the PFC response. In order to examine these aspects of immunoregulation, three studies were designed. In this chapter responses of rheumatoid B cells to autologous and allogeneic irradiated and non-irradiated rheumatoid and normal T cells were compared with the responses of normal B cells under the same conditions. In Chapter 10, soluble factors were harvested from the supernatants of allogeneic and autologous cultures and their effects on T cell-free B cell cultures studied. In Chapter 11

the effect of varying the proportions of T cells (irradiated and non-irradiated) to B cells was evaluated in rheumatoids and normals.

Allogeneic cultures necessitate the combination of genetically dissimilar cells. As was illustrated in Chapter 1, optimal communication between cells and subsequent responses are dependent upon the discrimination between self and non-self. Thus it was to be anticipated in this study that differences would exist between the responses in autologous cultures and those observed in cultures containing the constituent cell types in allogeneic cultures, quite apart from the presence of absence of disease. This was controlled for by running allogeneic normal-normal as well as allogeneic R.A. - normal culture combinations.

PATIENTS AND METHODS.

One patient with definite rheumatoid arthritis (DM) and two controls (nb, mm) were selected for study (Appendices 1 and 2). B and T lymphocytes from each individual were separated from 30 mls of fresh heparanized blood using density gradient centrifugation and nylon wool separation techniques previously described. The two normals were designated N.1 (nb) and N.2 (mm), and the rheumatoid designated R.A. (DM). Autologous cultures were prepared for each individual (BN_1 + TN_1, BN_2 + TN_2 and BRA+ TRA), as follows: $5x10^4$ B cells alone ($\pm$ PWM Lot. No. 15K0201), $5x10^4$ T cells alone ($\pm$ PWM), $5x10^4$ B cells and $5x10^4$ non-irradiated T cells ($\pm$ PWM), $5x10^4$ B cells + $5x10^4$ T_{3200} cells ($\pm$ PWM) and $5x10^4$ B cells + $5x10^4$ T_{6400} cells ($\pm$ PWM).

Using the same concentration of cells, allogeneic cultures were prepared. Rheumatoid B cells ($B_{R.A.}$) were cocultured separately with non-irradiated T cells, T_{3200} and T_{6400} cells ($\pm$ PWM) from control N_1 (TN_1). Then B cells from N_1(BN_1) were cocultured with the corresponding T cells from the patient with rheumatoid arthritis ($T_{R.A.}$). Allogeneic cultures of normal cells were prepared using the same cell concentrations and culture conditions. Thus B cells of normal N_2 (BN_2) were cocultured with T cells from N_1(TN_1) and B cells from N_1 (BN_1) coculturedwith T cells from N_2 (TN_2). Cell combinations were thereafter cultured and plaqued as described in Chapter 4.

RESULTS. (Original data- Appendix 3- 914-944)

In autologous cultures (Table 33), previous observations were confirmed. The absent PFC response from any T cell only culture and low responses from B cell only cultures indicated effective cell separation. In both rheumatoids and normals responses were enhanced by coculture of B cells with non-irradiated T cells. With irradiation (3200 rads) significant (greater than x 2) enhancement occurred in the two normals (6257 to 13766 and 3428 to 8228) but not in the rheumatoid (3159 to 3785). With higher levels of radiation (6400 rads) responses fell to be intermediate between those from B cells alone and B + T cocultures (N_1 = 4857, N_2 = 1047, RA = 1071). In all cases responses were higher in the presence than absence of 1% PWM.

In allogeneic cultures (Table 33), normal and rheumatoid B cells were capable of responding to helper signals from allogeneic T cells. It was evident, however, that with the exception of the rheumatoid, PFC responses from the same B cells were less when co-cultured with allogeneic rather than their own autologous T cells (N_1 = 3200 vs 6257, N_2 = 3086 vs 3428). This likely represented a feature of allogeneic cultures whereby definition cells were genetically dissimilar and did not communicate as efficiently. In the rheumatoid, a slightly greater response was observed in allogeneic coculture with TN_1 (3829) than in autologous culture with T_{RA} (3159), hinting at a possible deficiency of T help.

TABLE 33- PFC RESPONSE IN AUTOLOGOUS AND ALLOGENEIC CULTURES.

	PFC/10^6 PBL						
CELLS	AUTOLOGOUS			ALLOGENEIC			
CULTURED	BN_1+TN_1	BN_2+TN_2	$B_{RA}+T_{RA}$	$B_{RA}+TN_1$	BN_1+T_{RA}	BN_2+TN_1	BN_1+TN_2
B+PWM	236	213	1000	1000	236	213	236
B-PWM	143	0	143	143	143	0	143
T+PWM	0	0	0	0	0	0	0
T-PWM	0	0	0	0	0	0	0
B+T+PWM	6257	3428	3159	3829	2343	3086	3200
B+T-PWM	2429	1571	1367	1571	536	-	1786
$B+T_{3200}$+PWM	13766	8228	3785	7571	2874	11600	7543
$B+T_{3200}$-PWM	8357	2857	1500	2357	571	-	2714
$B+T_{6400}$+PWM	4857	1047	1071	1579	1000	1620	1464
$B+T_{6400}$-PWM	1571	500	786	857	214	-	1000

N_1 = nb

N_2 = mm

RA = DM

It was evident that while non-irradiated R.A. T cells provided some help, that R.A. T_{3200} cells produced no significant enhancement, resulting in a PFC response slightly less (3785) than that observed when these same cells were cultured in allogeneic fashion with non-irradiated TN_1 cells (3829) (Figure 38). Furthermore, when R.A. B cells were cocultured with T_{3200} N_1 cells (7571 PFC/10^6PBL), significant enhancement (apporximately x 2) occurred and was of a similar order of magnitude to that seen in autologous cultures of normal B+T_{3200} cells. Before the significance of this finding could be assessed it was necessary to evaluate comparable responses in the allogeneic cultures of BN_2+TN_1 and BN_1+TN_2. When TN_1 and TN_2 cells were irradiated (3200 rads) prior to coculture, PFC responses from BN_2 and BN_1 were significantly enhanced (3086 to 11600 and 3200 to 7543 respectively, i.e. greater than x 3 and greater than x 2). Thus it was observed that given the opportunity (adequate T help) the rheumatoid B cell had the capability to respond in this system in a manner indistinguishable from that of a normal B cell. This suggested that the B cell response was not restricted as measured by the PWM driven PFC response in the reverse hemolytic plaque forming cell assay.

Comparing responses from B+T_{3200} + PWM combinations from BN_1+T_{RA} and BN_1+TN_2 with their respective B+T+PWM combinations it was evident that while the irradiation (3200 rads) of normal T cells resulted in enhancement (3200 to 7543), little occurred when rheumatoid

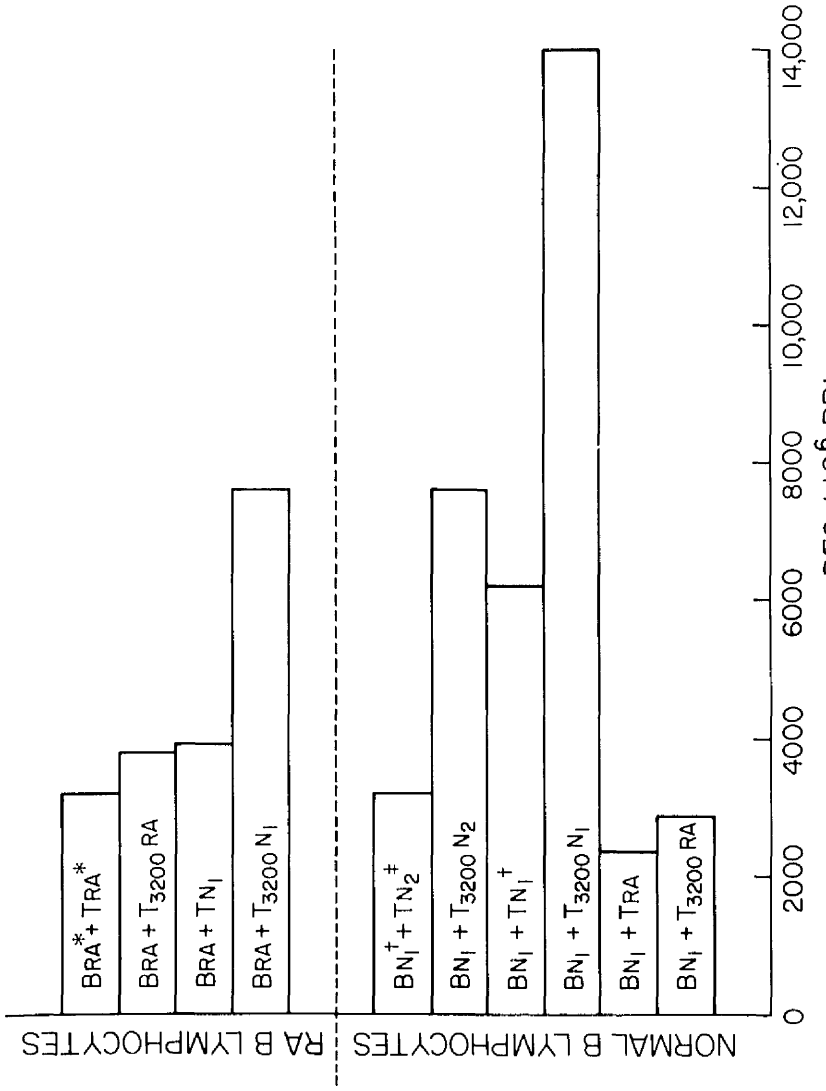


FIGURE 38 - AUTOLOGOUS AND ALLOGENEIC CULTURES (+PWM) OF B AND T LYMPHOCYTES FROM RHEUMATOID AND NORMAL SUBJECTS.

(N_1 = nb, N_2 = m, RA = rh)

T cells were similarly treated (2343 to 2874). Since the responding B cells were the same in both cases and since these cells were shown capable of radiation enhancement in both autologous (BN_1+TN_1) (6257 to 13766) and allogeneic (BN_1+TN_2) (3200 to 7543), normal-normal cultures it was concluded that T cell help in R.A. was restricted. Furthermore, when T_{RA} and TN_2 cells were irradiated at 6400 rads prior to culture, to destroy in addition T helper cells, responses fell less with the T_{RA} cultures (2874 to 1000) than with TN_2 cultures (7543 to 1464) further indicating a restriction of helper activity. Allogeneic cultures of normal cells (BN_2+TN_1 and BN_1+TN_2) were shown capable of significant radiation enhancement (3086 to 11600 and 3200 to 7543) at 3200 rads and significant declines (11600 to 1620 and 7543 to 1464) when cells were radiated at 6400 rads, confirming that the poor performance of rheumatoid cells could not be accounted for by the allogeneic nature of the system.

DISCUSSION.

The ability of rheumatoid and normal B cells to respond to allogeneic helper signals was demonstrated in this study. While these responses were not as great as those to autologous helper signals, it was shown that genetically dissimilar cells could communicate. Rheumatoid T help appeared restricted since (a) the allogeneic help from non-irradiated T cells was greater than the autologous help from non-irradiated rheumatoid T cells, (b) PFC responses from normal B cells was less in cocultures with allogeneic non-irradiated rheumatoid T cells than in cocultures with allogeneic normal or autologous T cells, (c) radiation enhancement by RA T_{3200} cells was minimal in both autologous and allogeneic cultures and (d) the relative loss of help was greater at higher levels of radiation when normal T cells were irradiated than when rheumatoid T cells were irradiated, as measured by the PFC response in both autologous and allogeneic cultures.

The response of rheumatoid B cells appeared adequate since coculture with allogeneic and autologous non-irradiated T cells resulted in augmentation of the PFC response, and was not restricted, because coculture with allogeneic normal irradiated (3200 rads) T cells resulted in a magnitude of enhancement seen in cultures containing normal but allogeneic cells.

Since this study was conducted on only a single combination of individuals, conclusions were limited. However, the findings of restricted RA T helper activity in the presence of potentially normal

RA B cell function were consistent with observations made in previous studies. Furthermore, since monocytes were predominantly found in the nylon wool adherent (B cell) fraction and played a role in the PWM driven PFC response it was reasonable to assume in RA B-normal T combinations, that RA monocytes and not normal monocytes (especially in irradiated T preparations) facilitated the response. Therefore, the observation that allogeneic (normal) T_{3200} cells enhanced the response of RA B cells, may indicate that RA monocytes were functioning normally in this system. However, since this was indirect evidence on a very small number of subjects it could not be regarded as conclusive.

SUMMARY.

The PFC response of rheumatoid and normal B lymphocytes to autologous and allogeneic helper signals were examined in this study. It appeared that in the few subjects studied the PFC response of rheumatoid B lymphocytes was intrinsically normal, while a defect existed in T helper activity. Indirect evidence suggested that RA monocyte function was adequate. In order to confirm these findings evaluations should be conducted on larger numbers of subjects.

CHAPTER 10

AUGMENTATION OF POLYCLONAL IMMUNOGLOBULIN SYNTHESIS BY SOLUBLE ALLOGENEIC AND AUTOLOGOUS HELPER FACTORS.

(STUDY 12)

- INTRODUCTION
- EXPERIMENT 1 - PATIENTS AND METHODS
  - RESULTS
  DISCUSSION
- EXPERIMENT 2 - PATIENTS AND METHODS
  - RESULTS
  DISCUSSION
- CONCLUSIONS
- SUMMARY

"He could by Euclid prove long sine
A ganging point compos'd a line
By numbers too he cou'd divine
When he did read
That three times three just made up nine."

Robert Ferguson (1750-1774)

INTRODUCTION:

The precise mechanism by which cells communicate and helper signals are transmitted remains to be elucidated. While direct cell-cell interaction may be one mode of communication, demonstration of the release in vitro of biologically active molecules from lymphocytes, suggests a definite role for communication by soluble factors.

Naskill et al[345] using murine cells demonstrated that thymus cells and some cell fractions could stimulate SRBC responses in other cell fractions, even when separated from them by a nucleapore or dialysis membrane. Although the response observed was not achievable with all fractions, these experiments suggested that cells could communicate by means other than by cell-cell interaction i.e. by soluble factors. Using a different model[346], it was demonstrated that a material extracted by gently heating peritoneal cavity cells from immunized mice, could substitute for the intact cells in a synergistic immune response with marrow cells. In separate experiments[347], in which T-B cell communication by a soluble factor was confirmed, it was noted that cell supernatants were inactive if they were obtained from cultures in which the antigen was omitted, indicating that antigen stimulation was a prerequisite for obtaining an active supernatant. At variance with Naskill's report this factor was not dialyzable. Soluble factors may also be of importance in

T-T cell interaction since they may substitute for T cells in T-T collaboration which results in the generation of T killer lymphocytes in mice[348].

Two distinct active helper moieties have been considered to exist, one nonspecific, and the other antigen specific. They may participate together, part or all of the time, in the development of antibody responses. Attempts to characterize the nonspecific moiety in mice[349] have suggested that it has a molecular weight of 30,000 - 40,000 and is capable of acting directly on B cells (in the presence of antigens) to effect triggering and subsequent differentiation and proliferation to antibody forming cells in vitro. The observation, that this enhancing factor could be removed by an immunoadsorbent prepared with an anti-Ia antiserum indicated that the biologically active moieties responsible for T/B cell interaction were the incorporated products of genes in the I region of the murine H-2 gene complex[350].

In contract to this nonspecific factor, Munro and Taussig[74] characterised an antigen-specific helper factor in mice. The factor was not an immunoglobulin, since it did not react with anti-Ig reagents. It had a molecular weight of approximately 50,000 , and contained within it Ia determinants. Thus like the nonspecific factor it appeared to be coded for by the I region of H-2 gene complex.

While the factors so far described have had helper activity, other factors having suppressor activities have been reported[351].

A vast array of soluble factors from lymphocytes and monocytes (lymphokines and monokines), have been shown capable in different systems of enhancing or suppressing responses of lymphocytes and monocytes. To preserve response specificity, it seems likely that some of these soluble factors share immunologic identity with cell surface molecules. Thus cell-cell interaction may occur between membrane-bound subunits with molecular similarity to soluble factors, or by the action of short radius factors, while more distant communication may be effected by the release of these or equivalent units as soluble factors, having a longer range of action. Apart from a facilitatory role, soluble factors may in themselves regulate the immune response. It has been observed that blastogenesis and proliferative responses of rodent cell cultures decline in the situation of overcrowding[352]. This type of phenomenon has been attributed to the release of inhibitory factors by lymphocytes which sense overcrowding[353]. It seems likely that short radius factors would be sufficient for these purposes.

There is a biologic necessity for an immune response, which can vary over a wide range but always be maintained in control. It has been suggested that modulation is achieved through feedback

loops, (possibly mediated by soluble factors), similar to those which regulate endocrine glandular function. The possibility of three levels of control has been considered[354], the first level being governed by specific mediator, the second by nonspecific mediator and the third by prostaglandins and cyclic nucleotides. It has been suggested that these mediators are bifunctional molecules, interacting specifically with antigen and with receptor sites on target cells, and furthermore that they may show multi-hit kinetics. Autoregulation has been recognized in several non-endocrine systems e.g. the control of cell mitosis by soluble suppressor factors, (Chalones) has been demonstrated in the homeostasis of human blood corpuscle production[355, 356].

While early studies were conducted on rodents, recent investigation in humans has shown that major histocompatability complex (MHC) gene products, encoded by the HLA-D region, play an important role in the generation and function of these soluble mediators, and in some instances comprise a portion of the helper molecules themselves[357, 358, 359]. However, immuno-precipitation studies suggest that at least some of these helper molecules do not bear Ia determinants[359]. Soluble factors which suppress immunoglobulin production in allogeneic and autologous cultures have been demonstrated in humans[360] and in the same laboratory the existence of non-T suppressor cells[298].

In recent experiments on human lymphocytes Chiorrazi et al[361, 362] have generated autologous and allogeneic helper factors from mixed lymphocyte cultures. These helper supernatants could be produced in the absence of antigen. Dose response curves constructed in a direct anti-SRBC PFC assay, indicated that the maximal response occurred at optimal concentrations of helper factors (33% volume-volume) and if higher doses were used the response declined. A small percentage of Ia^+ cells were demonstrated to be responsible for almost all the helper activity generated in allogeneic supernatants. Helper factors were generated by culturing T cells and irradiated (3,000 rads) B cells for 48 hours. Supernatants were then collected and filtered through a 0.45 micron millipore (Millipore, Bedford, Massachusetts, U.S.A.) filter and either immediately used or frozen at -70°C. The peak response observed when factors were then added to B cell cultures, usually occurred at factor concentrations between 25 and 50%.

While lymphokine release has been observed in rheumatoids, problems of definition and characterization of the constituent molecular entities has hindered progress in the field. In the study which follows an attempt was made to determine whether soluble helper factors were released from rheumatoid cultures, whether they could substitute for T lymphocytes, what the optimal working concentrations were, and whether in allogeneic and autologous systems they were more or less potent than factors from control cultures.

It was postulated, that a failure of rheumatoid T cells to release soluble helper factor or of rheumatoid B cells to such helper signals might provide an explanation for radiation enhancement failure in rheumatoids.

PATIENTS AND METHODS.

Nine patients with classic or definite rheumatoid arthritis and nine normal controls were selected for study (Appendices 1 and 2). Two types of experiments were devised. Experiment No. 1 examined the variation in the responses of B cells to different helper factors in rheumatoids and normals. Experiment No. 2 evaluated dose response curves for soluble factors and the effects of T cell pre-irradiation on soluble factor generation.

Experiment No. 1

Subjects and Methods:

Six patients with classic or definite rheumatoid arthritis (MS, MS_1, MB, JC, RR and NI - Appendix 1) and nine normal controls (jd, mf, nb, cm, db, jo, cp, bw and ch - Appendix 2), were selected for study.

Autologous factors were raised in three rheumatoids (MS_1, MB and JC) and five normals (bp, bw, ch, cm and mf). Lymphocytes were separated from fresh heparanized blood by the density gradient centrifugation and nylon wool separation techniques previously described. B cells were irradiated at 3,000 rads (B_{3000}), following which they were washed three times in RPMI 1640. $5x10^4$ B_{3000} and $5x10^4$ T cells were cocultures in replicate for 48 hours at 37^oC in 1 ml of RPMI 1640 (containing L-glutamine) supplemented with 10%

heat inactivated sheep red blood cell absorbed foetal calf serum (Grand Island Biological Company, Grand Island, New York) and gentamicin 15 micrograms per ml (Schering Corporation, Kenilworth, New Jersey 07033). At the end of this period supernatants were extracted with a 1 ml syringe, filtered through a 0.45 micron millipore filter (Millipore, Bedford, Massachusetts, U.S.) and either used immediately or frozen. Since factors from different cultures of the same cells may have differed somewhat, supernatants from replicate cultures were pooled.

Autologous cultures of B cells alone, B+T cells and $B+T_{3200}$ cells in 1:1 ratio combinations were established as previously described. Cells were cultured in 300 microlitre volumes of either a mixture of one third factor to two thirds 10% heat inactivated sheep red blood cell absorbed foetal calf serum in RPMI 1640, or in 10% heat inactivated sheep red blood cell absorbed foetal calf serum in RPMI 1640 only, (supplemented with gentamicin, as above) for seven days at $37^{\circ}C$ in the presence of 1% pokeweed mitogen (Lot No. 12N5302). Following incubation for 7 days, cells were washed and plaqued as previously described. Results were expressed as $PFC/10^{6}$ PBL.

RESULTS.

Mean responses for rheumatoids and controls are tabulated in Tables 34 and 35. Four groups can be identified:

A. Cultures of normal lymphocytes with normal factors.

B. Cultures of normal lymphocytes with rheumatoid factors.

C. Cultures of rheumatoid lymphocytes with normal factors.

D. Cultures of rheumatoid lymphocytes with rheumatoid factors.

In each of the groups it was evident in the absence of soluble factors, that the PFC response was enhanced by the addition of T cells to B cell cultures. As had been previously noted, responses were higher in $B+T_{3200}$ than B+T cultures in normals but no significant radiation enhancement occurred in rheumatoids. With one exception [cm + $F(MS_1)$ = 2850], the PFC response from each culture type (B, B+T and $B+T_{3200}$) was greater in the presence of factor. It was evident in A, B and C that different factors produced different PFC responses from the same cells. Thus the helper activity of supernatants from different individuals (rheumatoids and controls) varied in their effects on the same B cell cultures e.g_1 B+T(jd) + F(mf) = 3150, B+T(jd) + F(bw) = 1650. e.g_2 B+T(db) + F(JC) = 15200, B+T(db) + F(MB) = 7400.

Similarly the same factors had different effects on different cells. Thus, although supernatants from JC increased the response of db's B+T cultures from 2400 to 15,200, they could only increase

TABLE 34 - PFC RESPONSES OF NORMAL LYMPHOCYTES IN CULTURES CONTAINING ALLOGENEIC NORMAL AND RHEUMATOID SOLUBLE HELPER FACTORS.

			PFC/10^6 PBL		
	CELL SOURCE	FACTOR SOURCE (33%)	B	B + T	B + T3200
(A)	j.d.	-	0	1250	2950
		n.f.	500	3150	4150
		b.w.	850	1650	3350
	m.f.	-	50	3100	5800
		c.p.	1850	4600	7000
	n.b.	-	260	3960	7200
		c.h.	11600	-	-
(B)	j.d.	-	0	1250	2950
		M.B.	50	1650	3400
		$M.S._1$	0	1850	3150
	c.m.	-	0	1150	2860
		M.B.	50	1400	3800
		$M.S._1$	700	1750	2850
	m.f.	-	50	3100	5800
		M.B.	2500	3300	6200
		$M.S._1$	4100	6400	6500
		J.C.	1400	5400	6500
	n.b.	-	260	3960	7200
		$M.S._1$	6800	4600	11760
	d.b.	-	1700	2400	9700
		J.C.	2600	15200	18600
		M.B.	4800	7400	14450
	j.o.	-	0	16950	26200
		J.C.	26800	31750	40750
		M.B.	10800	18100	32100

TABLE 35 - PFC RESPONSES OF RHEUMATOID LYMPHOCYTES IN CULTURES CONTAINING ALLOGENEIC RHEUMATOID AND NORMAL SOLUBLE HELPER FACTORS.

			PFC/10^6 PBL		
(C)	CELL SOURCE	FACTOR SOURCE (33%)	B	B + T	B + T3200
	M.S.	-	200	2250	2750
		o.h.	2200	5900	6050
		o.m.	2000	11900	11900
	N.I.	-	500	4300	4150
		o.h.	2400	4650	4350
		o.m.	1000	4550	4800
	R.R.	-	175	4050	3800
		o.p.	2000	4650	7000
		b.w.	1150	6100	6700
(D)	R.R.	-	175	4050	3800
		J.C.	3600	5500	5700

the response of mf's from 3100 to 5400. This was not a unique feature of rheumatoid factors, since factors from ch more than doubled MS's B+T response, but had little effect on similar cultures from subject NI.

It was evident that some rheumatoid factors produced greater responses than normal factors, i.e. factors from MS_1 and JC but not those from MB produced greater responses (6400, 5400 and 3300 respectively) from B+T cultures of control subject mf, than did factors from normal subject cp (4600).

Factor enhancement was calculated for each type of culture in the four groups by dividing PFC response with factor by PFC response without factor (Table 36) .

$$\text{i.e. Factor Enhancement} = \left[\frac{\text{PFC response in presence of factor}}{\text{PFC response in absence of factor}} \right]$$

$$\text{e.g. Factor Enhancement for } B+T(jd) + F(mf) = \frac{3150}{1250} = 2.52$$

It should be noted that factor enhancements of∞ were not included in the calculation of mean enhancement (only B alone cultures affected), since enhancement in these instances was determined by zero response in cultures not containing factor, rather than by any specific effect of such factors.

TABLE 36 - FACTOR ENHANCEMENT BY SOLUBLE HELPER FACTORS IN RHEUMATOID AND NORMAL SUBJECTS

	CELLS / FACTOR	FACTOR ENHANCEMENT IN CULTURE COMBINATIONS (= LYMPHOCYTES + FACTOR / LYMPHOCYTES - FACTOR)		
		B	B + T	B + T3200
A.	jd/mf	∞	2.5	1.4
	jd/bw	∞	1.3	1.1
	mf/bw	37	1.5	1.2
	nb/ch	45	-	-
	MEAN	41	1.8	1.2
B.	jd/MB	∞	1.32	1.1
	jd/MS[1]	0	1.5	1.1
	cm/MB[1]	∞	1.2	1.3
	CM/MS[1]	∞	1.5	0.9
	mf/MB[1]	50	1.1	1.1
	mf/MS[1]	82	2.1	1.1
	mf/JC[1]	28	1.7	1.1
	nb/MS[1]	26	1.2	1.6
	db/JC[1]	1.5	6.3	1.9
	db/MB	2.8	3.1	1.5
	jo/JC	∞	1.9	1.6
	jo/MB	∞	1.1	1.3
	MEAN	27	2.0	1.3
C.	MS/ch	11	2.6	2.2
	MS/cm	10	5.3	4.3
	NI/ch	4.8	1.1	1.1
	NI/cm	2	1.1	1.2
	RR/cp	11.5	1.2	1.8
	RR/bw	6.6	1.5	1.8
	MEAN	8	2.1	2.1
D.	AR/JC	21	1.4	1.5

It was evident that a range of enhancement existed for each of the three culture combinations in A, B and C, category D being represented by only single data points. Mean factor enhancements for each of the categories A, B, C and D were 41, 27, 8 and 21 for B cells only, 1.8, 2.0, 2.1 and 1.4 for B+T combinations and 1.2, 1.3, 2.1 and 1.5 for B+T_{3200} combinations respectively. It was evident that factors had a much greater effect when added to cultures lacking any alternative source of T cell help (i.e. B cell only cultures) than when added to cultures containing T or T_{3200} cells. This effect was independent of the source of the factor or of the B cell. No significant difference existed between the enhancing effect of factors in B+T versus B+T_{3200} cultures, both being in the range x1 to x2. While it was true, that in general the enhancing effect on B+T cultures was greater than that on B+T_{3200} cultures for each situation considered, consistent with the likelihood that the percentage addition of help was smaller in B+T_{3200} cultures, there were several exceptions.

DISCUSSION.

Rheumatoid cultures were able to generate helper factors capable of enhancing the PFC response of B cells in a variety of normal and rheumatoid cultures to a similar extent to that which factors from normal control cultures were able to enhance the PFC response of B cells in other normal and rheumatoid cultures. This suggested that not only were rheumatoid T cells capable of generating

potent helper factors in this system, but also that rheumatoid B cells could normally respond to soluble helper signals. In 27% of cases, the PFC response produced by the addition of factor (but not non-irradiated T cells) to B cell cultures, was greater than that produced by the addition of non-irradiated T cells (but not factor). In these instances it was evident that in the concentration used the B cell response to a soluble factor from an allogeneic individual was greater than that to the patient's own autologous T cells.

Experiment No. 2.

Patients and Methods.

Three patients with rheumatoid arthritis (LL, ML and SW) and one control (db) were selected for study. Lymphocytes were separated from fresh heparanized blood by the density gradient centrifugation and nylon wool separation methods previously described. The cells were washed three times in RPMI 1640 following which B cells were irradiated at 3000 rads. They were subsequently washed three times in RPMI 1640. $5x10^4$ irradiated B cells (B_{3000}) were cocultured with $5x10^4$ T cells (either non-irradiated or T_{3200}) for 48 hours at 37^oC in 1 ml RPMI 1640 (containing L-glutamine) supplemented with 10% heat inactivated sheep red blood cell absorbed foetal calf serum (Grand Island Biological Company, Grand Island, New York) and gentamicin 50 micrograms per ml (Schering Corporation, Kenilworth,

New Jersey 07033). Cells were cultured in the absence of pokeweed mitogen. Autologous cultures were set up in replicate in order to generate an adequate volume of soluble factor. After 48 hours supernatants were extracted with 1 ml plastic syringe, filtered through a 0.45 micron millipore filter (Millipore, Bed., Massachusetts, U.S.), pooled (across replicate cultures) and either used immediately or frozen.

Dose-response determination.

In order to evaluate the potency of these factors cultures of $5x10^4$ B cells alone, $5x10^4$ B cells with $5x10^4$ non-irradiated T cells and $5x10^4$ B cells + $5x10^4$ T_{3200} cells were established as previously described in study No. 6. Cells were cultured for a duration of 7 days in the presence of 1% pokeweed mitogen (Lot No. 12N5302). However, in contrast to study No. 6 cells were cultured in 0.3 ml(300µl) volumes of RPMI 1640 (containing L-glutamine) supplemented with 10% heat inactivated sheep red blood cell absorbed foetal calf serum and gentamicin 50 micrograms per ml. In this 300 microlitre volume however, helper cell factors in some cultures replaced a variable (0-100%) proportion of the 10% FCS in RPMI 1640 used as a culture medium. These smaller culture volumes were necessary as the supply of helper factor was limited. Cells were cultured for a period of 7 days under the conditions previously described, then washed and plaqued. Results were expressed as PFC/10^6 viable PBL.

RESULTS.

The results of experiments conducted with two types of autologous factor (F1 = B_{3000} + T and F2 = B_{3000} + T_{3200}) are represented in Tables 37-40. In Table 37 autologous factors were generated in the same rheumatoid individual in whom they were tested in cultures of B cells alone, B + non-irradiated T and B + T_{3200} cells. In the absence of F1 a response of 1320 PFC/10^6PBL was obtained (i.e. from B cells alone). This was higher than reported in previous chapters and reflected the higher responses obtained with this Lot of pokeweed mitogen. When non-irradiated T cells were added, the PFC response was enhanced (7384), but with irradiation of T cells (T_{3200}) further enhancement was minimal (8490), consistent with previous observations in other rheumatoids. When B cells were cultured alone with F1 a dose dependent response was observed being maximal at 40%. A second lesser peak was observed at 70% with a decline in between these values. It was evident that both high and low concentrations of factor resulted in submaximal responses. Thus autologous factors were capable of substituting for the presence of T cells and could enhance the PFC response of autologous B cells. The maximum response to F1 was greater than that observed when B cells were cultured with T or T_{3200} cells. Furthermore, cultures containing 33% F1 produced responses from B, B+T and B+T_{3200} cultures above those achieved without F1, suggesting that these

TABLE 3% - DOSE RESPONSE ASSOCIATION BETWEEN B LYMPHOCYTES FROM RHEUMATOID SUBJECT M.L. WITH AUTOLOGOUS SOLUBLE HELPER FACTORS.

CELLS (ML)	%FACTOR (F1)	PWM	PFC/10^6PEL	%FACTOR (F2)	PWM	PFC/10^6PEL
B	-	+	1320	-	+	1320
B	10%	+	1600	10%	+	17593
B	20%	+	1000			
B	33%	+	4218	33%	+	17100
B	40%	+	21978			
B	50%	+	14442	50%	+	22400
B	60%	+	13778			
B	70%	+	18500	70%	+	21945
B	100%	+	9711	100%	+	13005
B	-	+	1320			
B + T	-	+	7384			
B+T3200	-	+	8490			
B	33%	+	4218	33%	+	17100
B + T	33%	+	21500	33%	+	18315
B+T3200	33%	±	9940	33%	+	35250

F1 = FACTOR FROM B_{3000} + T - PWM CULTURES OF SUBJECT M.L.
F2 = FACTOR FROM B_{3000} + T_{3200} - PWM CULTURES OF SUBJECT M.L.

TABLE 38.- DOSE RESPONSE ASSOCIATION BETWEEN B LYMPHOCYTES FROM NORMAL SUBJECT d.b. AND AUTOLOGOUS SOLUBLE HELPER FACTORS.

CELLS (db)	%FACTOR (F1)	PWM	PFC/10^6PBL	%FACTOR (F2)	PWM	PFC/10^6PBL
B	-	+	1200			
B + T	-	+	24568			
B+T3200	-	+	35524			
B	10%	+	19250			
B	20%	+	11218			
B	33%	+	9940	33%	+	13800
B	40%	+	9130			
B	50%	+	7750			
B	60%	+	10224			
B	70%	+	13250			
B	90%	+	10200			
B	100%	+	6972			
B + T	33%	+	28860	33%	+	33600
B+T3200	33%	+	4250	33%	+	33366

F1 = FACTOR FROM B_{3000} + T - PWM CULTURES OF SUBJECT d.b.

F2 = FACTOR FROM B_{3000} + T_{3200} - PWM CULTURES OF SUBJECT d.b.

TABLE 39 - DOSE RESPONSE ASSOCIATION BETWEEN B LYMPHOCYTES FROM NORMAL SUBJECT d.b. AND ALLOGENEIC SOLUBLE HELPER FACTORS FROM RHEUMATOID SUBJECT S.W.

TABLE HELPER FACTORS FROM RHEUMATOID SUBJECT S.W.

CELLS (db)	%FACTOR (F1)	PWM	PFC/10^6PBL
B	10%	+	1162
B	20%	+	3550
B	33%	+	4600
B	40%	+	6500
B	50%	+	6250
B	60%	+	6000
B	70%	+	6750
B	80%	+	12500
B	90%	+	2556
B	100%	+	2500

F1 = Factor from B_{3000} + T - PWM cultures of subject S.W.

TABLE 49 - DOSE RESPONSE ASSOCIATION BETWEEN B LYMPHOCYTES FROM RHEUMATOID SUBJECT L.L. AND AUTOLOGOUS SOLUBLE HELPER FACTORS.

CELLS (LL)	%FACTOR (F1)	PWM	PFC/10^6PBL	%FACTOR (F2)	PWM	PFC/10^6PBL
B	-	+	999			
B	5%	+	1170			
B	10%	+	9373			
B	20%	+	16576			
B	30%	+	11880			
B	40%	+	7700			
B	50%	+	10500			
B	60%	+	13750			
B	70%	+	9316			
B	80%	+	4845			
B	90%	+	6900			
B	100%	+	5112			
B	33%	+	14630	33%	+	22684
B + T	33%	+	9680	33%	+	11172
B+T3200	33%	+	17514	33%	+	37200
B + T	-	+	4260			
B+T3200	-	+	20217			

F1 = Factor from B_{3000} + T - PWM cultures of subject L.L.

F2 = Factor from B_{3000} + T_{3200} - PWM cultures of subject L.L.

factors had significant potency. F2 factors derived from 48 hour B3000 + T_{3200} cultures produced greater enhancement (with one exception), than F1 factors derived from B_{3000} + T cultures, indicating that the net activity of soluble factors was the result of a balanced secretion of helper and suppressor factors or alternatively that radiation facilitated the secretion of increased quantities of helper factor. However, there were some inconsistencies in these results in that while B + T_{3200} + F2 was greater than B + F2, B + T_{3200} +F1 was less than B + T + F1. Furthermore, the PFC response of B + T + F2 was only slightly greater than B + T + F2 while B + T + F1 was much greater than B + F1. This may have reflected an effect of different concentrations of soluble factors, or have been a result of not conducting determinations on replicate cultures for each condition (i.e. the observed response was not representative).

Table 38 shows responses for autologous factors F1 (B_{3000} + T supernatants) and F2 (B_{3000} + T_{3200} supernatants) from normal subject db, when used to supplement his own cultures of B cells, B + T cells and B + T_{3200} cells. The response from B cells alone (1200) was enhanced by the addition of T cells (24568) and maximal in the presence of T_{3200} cells (35,524). Soluble factors significantly enhanced the response at all concentrations (6972 to 19250) and F2 enhanced the response more than equivalent concentrations (33%) of F1 at the three points of comparison (13,800 vs 9,940, 33,600 vs 28,860 and 33,366 vs 4,250). However, responses from B + T_{3200} + F (1 or 2) were less than

from B + T + F (1 or 2) cultures.

Table 39 displays responses for autologous factor F1 (B_{3000} + T supernatants) from rheumatoid subject SW when used to supplement cultures of B cells from normal subject db. Responses from factor supplemented B cell only cultures, ranged from 1162 to 12,500, the maximal response (12,500) occurring at a factor concentration of 80%.

Table 40 displays responses from autologous factors F1 (B_{3000} + T supernatants) and F2 (B_{3000} + T_{3200} supernatants) from rheumatoid subject LL when used to supplement her own B, B+T and B+T_{3200} cell cultures. The responses from B cells alone (999) was enhanced by the addition of T cells (4260) and further enhanced by the presence of T_{3200} cells (20,217). Soluble factors significantly augmented the response at all concentrations (1170 to 16,576) and F2 enhanced responses more than equivalent concentrations of F1 at the three points of comparison (22684 vs 14630, 11,172 vs 9680 and 37200 vs 17514).

Certain generalizations were made from these results. In all instances responses from B cell only cultures were greater in the presence than absence of factors, suggesting that these factors could indeed substitute for the presence of T lymphocytes. The filtered supernatants from B_{3000} + T_{3200} cell cultures enhanced the B cell response greater than did those from B_{3000} + T cultures,

indicating the greater net helper activity of factors generated by these irradiated T cells.

Dose response profiles (Figure 39) constructed for the above combinations, showed marked irregularity, with no distinct pattern emerging. It appeared that soluble factors from rheumatoids and normals enhanced the PFC response of B cells at various concentrations and to various degrees. A tendency for responses to decline at the extremes of factor concentrations could be appreciated inspite of the sawtooth profile of these response curves.

DISCUSSION:

These results confirmed the previous findings that autologous helper factors could be raised in both rheumatoids and normals and that these factors could substitute for the presence of T lymphocytes. Supernatants from cultures containing irradiated T cells had greater helper activity than those from cultures containing non-irradiated T cells. The inability to define smooth dose response curves likely reflected the effects of between-plaque and between-culture variation. The only suggestion of deficient rheumatoid T help was that the profile responses of lymphocytes from normal subject db, were lower in the presence of factor from rheumatoid subject SW than with his own autologous factors. However, the limited number of data points and the problem of making comparison between the autologous and allogeneic conditions in which this difference was

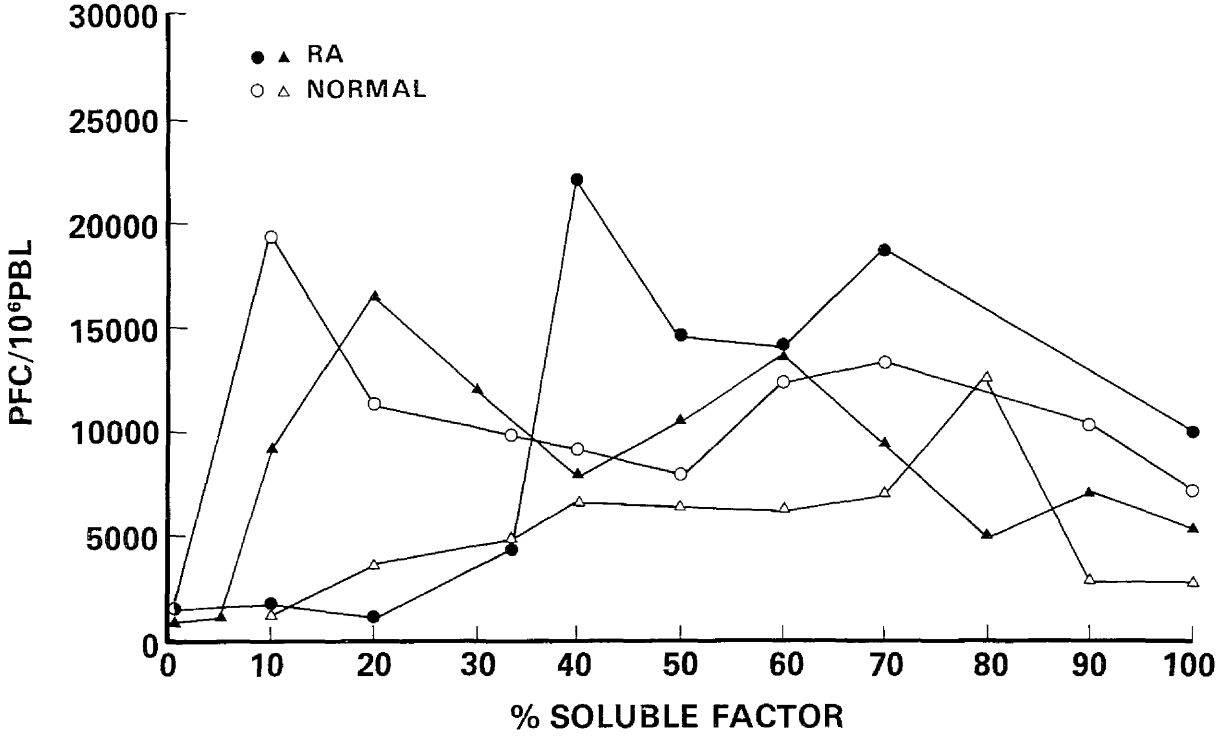


FIGURE 39 - DOSE RESPONSE CURVES OF B LYMPHOCYTES TO SOLUBLE HELPER FACTORS IN R.A. AND NORMAL.

● = ML/(F)ML, ▲ = LL/F(LL), ○ = db/F(db), △ = db/F(SW)

observed, prohibited any real conclusion being drawn. The decline in PFC response at high factor concentrations was consistent with observations made by other researchers. While this had formerly been demonstrated in normal subjects it also appeared to be a feature of rheumatoid cultures. Finally, variation in the mitogenic activity of different batches of pokeweed mitogen was demonstrated (15K0201 vs 12N5302).

CONCLUSION.

These studies have validated a method for raising soluble helper factors from autologous culture systems. While allogeneic cultures of rheumatoid and normal lymphocytes were also established and shown capable of generating helper factors, experiments were only conducted on a small scale and therefore were not presented here.

In the first experiment of this study, autologous factors from rheumatoids were shown to be capable of crossing allogeneic boundaries and enhancing the PFC response from B cells of other rheumatoids and also of controls. There was no deficiency in the potency of these factors, a wide range being described in both patients and controls. In view of the wide range of PFC responses from the same cultures with different factors, and from different cultures with the same factors, it was not surprising that between group differences were absent. The greater enhancement seen when factors were added to

B cell only cultures, contrasted with their lesser effect when added to B + T or B + T_{3200} cultures. This difference was likely related to the fact that the increment in total help as a consequence of adding "extrinsic" help (i.e. factors)to "intrinsic" help (i.e. T cells) was less in the latter cultures. In accordance with the observations of other workers, PFC responses levelled off or even declined after a certain point as more factor was added in both normal and rheumatoid cultures.

In experiment No. 1 factors were allogeneic to the cultures in which they were used. It was of interest therefore that a 33% concentration of these factors, produced greater enhancement in 27% of cases, than the addition of autologous non-irradiated T cells. This apparent paradox may have been a consequence of conducting too few replicate determinations for each lymphocyte/ factor combination, since with these same lymphocytes but other soluble factors, the converse was true. In experiment No. 2 it was demonstrated that the response was dose dependent in a manner which was not predictable for either normal subjects or rheumatoids. Thus the choice of a factor concentration of 33% in experiment No. 1 may have resulted in the use of factor at other than optimal concentrations.

While evidence indicated that T cells could communicate with B cells (autologous and allogeneic, rheumatoid and normal) by soluble factors, and therefore that cell-cell contact was not

essential, the mechanism of this communication was not examined. The effects of factors generated in the supernatants of B+T cultures (intrinsic) were not strictly comparable to the effects of these same factors when harvested and used as extrinsic factors in the supplementation of B cell only cultures since:

A. Any effect that B cells had on the T cell factor produced may have differed since to generate helper factor, B cells were irradiated.

B. Factor activity may have varied with culture duration. Thus to generate extrinsic factors cells were cultured for 2 days, while in the normal situation cultures were incubated for 7 days.

C. Intrinsic factor was undiluted in the regular cultures, while it was used at a 33% concentration when added in an extrinsic manner.

D. Factors were raised in the absence of pokeweed mitogen.

These harvested supernatants likely contained several different soluble factors of varying molecular weight, Ia positivity, helper and suppressor activity and potency. Thus these studies only allowed an examination of the net effect of the factors in one in vitro situation and at one point in time. Furthermore, evidence from experiment No. 2 indicated that the potency of supernatants from

B_{3000} + T_{3200} cultures was greater than that from comparable B_{3000} + T cultures, suggesting a shift in the relative secretion of helper and suppressor factors resulting in an alteration in net activity, which favoured help. Thus while these experiments provided some insight into the generation and activity of soluble factors, they lacked the necessary sophistication required to dissect the role of these factors in immunoregulation, define the spectrum of such factors, or characterize their individual regulatory activities.

Although there was some indication of restricted T cell help in the experiment No. 2, response variation was such for any given lymphocyte combination or factor that the detection of small but significant between group differences was prohibited. As far as could be judged from these studies, rheumatoid T lymphocytes (under the in vitro conditions existing in the two experiments), were capable of releasing potent soluble factors, having a net helper activity similar to that of factors released from normal lymphocytes. However, detection of significant between group differences would necessitate a large scale study controlling for inherent test variability. In agreement with the last study, (Chapter 9) rheumatoid B lymphocytes were shown to be normally responsive to adequate helper signals.

Thus it was suspected, that the failure of radiation enhancement in rheumatoids was due to a deficiency in the function of helper T cells, either as the only defect or in combination with the dysfunction of suppressor T cells. One final study was planned to examine the effects of increasing numbers of lymphocytes in cultures containing B + T cells, in order to further evaluate RA B cell responsiveness and the ability of increased quantities of T cells to overcome the suspected qualitative defect.

SUMMARY:

These experiments demonstrated that a modification of a previously described method could be successfully used to generate soluble factors not only from cultures of lymphocytes from normal individuals, but also from those of patients with rheumatoid arthritis. While there were some methodologic restrictions, it was apparent that potent soluble helper factors could be derived in this manner and that their activity crossed allogeneic boundaries. Although a dose response relationship was shown, and was in part consistent with the previous observations in that excessive concentrations of helper factors paradoxically resulted in a decline in the B cell response, the profiles described were quite irregular. While this irregularity may have been in part related to the study method, it may also have

indicated the existence of a complex relationship between soluble factors and cellular response. It seems important therefore that in studies of this kind some uniformity be achieved in the potency of factors used, and that experimental conditions be rigorously standardized and appropriate controls established. Furthermore, if the factor concentration for peak response varies between factors, then the complexity of experimentation is necessarily increased. The method described, allowed examination of the net effect of helper and suppressor soluble factors, both of which were almost certainly present in the culture supernatants. Thus, consistent with other observations, the irradiation of T cells prior to coculture with irradiated B cells resulted in the generation of soluble factors having greater helper potency. It was evident, that more sophisticated techniques were required to appreciate the complexity of communication between cells via these biologically active molecules. However, this study indicated, that under certain experimental conditions, rheumatoid T cells were capable of producing potent factors having net helper activity similar to that produced by factors from normal T cells. Finally, responsiveness of rheumatoid B cells to allogeneic normal and allogeneic rheumatoid T cell signals was observed to be normal.

CHAPTER 11

FUNCTIONAL ANALYSIS OF HUMAN T CELL SUBSETS USING DIFFERING T/B CELL RATIOS (STUDY 13)

- INTRODUCTION
- PATIENTS AND METHODS
- RESULTS
- DISCUSSION
- SUMMARY

"It is better to travel with
anticipation than to arive."

FRIDTJOF NANSEN 1861-1930.

INTRODUCTION.

The experiments conducted so far indicated the existence of a functional defect at the level of the T cell (or possibly monocyte). In irradiation and Tγ depletion studies the PFC response was restricted in rheumatoids. Tγ and OKT5+ enumeration studies suggested that the number of suppressor cells was normal and OKT4+ enumeration studies suggested that the number of helper cells was also normal in rheumatoid cultures. In allogeneic and factor studies, B cells were shown capable of normal responses when provided with appropriate helper signals. In addition these studies indirectly suggested functional normality of monocytes which were known to be present in normal numbers (non-specific esterase staining experiments).

Recent studies have allowed insight into some of the relationships which exist between T cell subsets identified by different methods and the ways in which T cells effect the PFC response of B cells. Thomas et al[363] used monoclonal antibodies and an RHPFC assay, to examine T cell interaction in the context of PWM-triggered human B cell differentiation in normal human lymphocytes. Functionally distinct T cell subsets were isolated by complement mediated lysis using monoclonal antibodies OKT4 (anti-helper) and OKT8 (anti-suppressor). Graded numbers of either untreated or irradiated (1250R) T cells were added to autologous B cell cultures and

Immunoglobulin synthesis measured on day 5 or 6 with a reverse hemolytic plaque forming assay. They observed that:-

1. As few as 1% T cells were required for significant PFC formation.
2. That the helper capacity of these small numbers of T cells (i.e. low T/B ratio) was radiosensitive. i.e. T non-irradiated cells were more effective than T 1250 cells in inducing B cell differentiation.
3. Only at high T/B ratios did PFC response with B+T1250 cells exceed that from B+T cells. These observations suggested the existence of more than one helper T cell subset, the presence of radiosensitive T helper cells, and a spectrum of radiosensitivity for this T cell subset.
4. OKT4 and OKT8 mediated help and suppression were shown to be dose dependent although the helper response declined above a certain level (0.4×10^6 cells).
5. Irradiation of OKT8+ cells resulted in a loss of suppression suggesting that the suppressor cell was radiosensitive.
6. Of greatest importance was the observation that when OKT8 positive (suppressor) cells were added to B cell cultures in the presence of irradiated OKT4+ cells, no suppression of the PFC response occurred. This suggested that suppression of the PFC response by OKT8+ cells was mediated by OKT4+ helper cells. Thus suppressor activity appeared to require collaboration between two distinct populations of radiosensitive cells, one residing in the OKT4+ population and the other in

the OKT8+ population. How this suppression is transmitted is unknown. However, OKT8+ cells have been shown capable of suppressing soluble helper factor release from OKT4+ cells but not in suppressing the helper activity of preformed factors even in the presence of OKT4+ cells [364]. This suggests that suppression is mediated through inhibition of the release of helper factors from T helper cells and not by a direct effect of suppressor T cells on B cells.

7. While T help could be diminished by optimal dose radiation at low T/B ratios, it was shown that at high T/B ratios help was radioresistant.

The complexity of immunoregulatory events is increased by indications that within the OKT4+ population exist subsets of helper cells. Using a different technique Reinherz et al[365], defined two populations of helper T cells, OKT4+ Ia+ and OKT4+ Ia-. While only the Ia+ subset produced a non-specific helper factor, both Ia+ and Ia- subsets were required to achieve maximum immunoglobulin production by B cells in an antigen driven system. Thus it is clear that not only do helper cells and suppressor cells interact but also that interaction occurs between subsets ofhelper cells (and likely between subsets of suppressor cells).

While these techniques have been applied to the study of normal subjects in the last 12 months, they have not previously been applied to the study of patients with rheumatoid arthritis. While a shortage of monoclonal antibodies prohibited the execution of experiments with pure populations of T cell subsets, the effect

of adding increasing numbers of T cells to B cells was studied in rheumatoids and normals. Furthermore, the definition of a population of radiosensitive T helper cells was sufficiently interesting to prompt an attempt at verification in normals and simultaneous application in the evaluation of rheumatoids.

PATIENTS AND METHODS:

Five patients with classic or definite rheumatoid arthritis (Appendix 1 - MK$_1$, VC, JG, EL and LL) and 16 normal subjects (Appendix 2 - db, db$_1$, mm, ma, cm, mf, jd, nl, ry, ph, me, hk, jw, cc$_1$, bt and bw) were selected for study. T and B lymphocytes were separated from 30 mls of fresh heparanized blood by the Ficoll Hypaque density centrifugation and nylon wool separation techniques previously described. T cells were either non-irradiated or irradiated at one of two dosages (1250 rads and 3200 rads) and washed three times in RPMI 1640 prior to coculture with B cells. In separate cultures a constant number of B lymphocytes ($5x10^4$) were cultured either alone or with numbers of T non-irradiated, T_{1250} or T_{3200} cells varying between $0.25x10^4$ and $40x10^4$. Cells were incubated for 7 days at 37°C in 1ml of RPMI 1640 (containing L-glutamine) supplemented with 10% heat inactivated sheep red blood cell absorbed fetal calf serum (Grand Island Biological Company, Grand Island, New York), and gentamicin 50 micrograms

per ml (Schering Corporation, Kenilworth, New Jersey 07033) in the presence of 1% v/v pokeweed mitogen Lot No. 12N5302. Following incubation, cells were washed three times and plaqued as previously described. Plaques were counted and expressed as PFC per 10^6 viable PBL.

RESULTS:

Individual (means derived from plaquing single cultures in replicate) PFC responses for selected T (non irradiated T1250, T3200) / B ratio combinations are displayed in Tables 41-43 (normal subjects) and Tables 44-46 (rheumatoid subjects). The overall high responses in this study reflected the use of a different batch of pokeweed mitogen as previously described. When considered on an individual basis it was evident that not only did responses vary between individuals for any given T/B ratio but also that peak responses (underlined) from a given normal subject frequently occurred at different T/B ratios between T non-irradiated, T_{1250} and T_{3200} cultures, e.g. while b.t's peak B + T non-irradiated response (17,300) occurred with $1x10^4$ T cells, the peak response with T_{1250} cells (16,400) occurred at $2.5x10^4$ cells and with T_{3200} cells (19,250) at $5x10^4$ cells. While less than 50% of peak responses with non-irradiated T cells occurred at $5x10^4$ cells, the majority of peak

TABLE 41 - EFFECT ON PFC RESPONSE OF VARYING THE T:B RATIO IN CULTURES CONTAINING B^{*} + T NON-IRRADIATED CELLS IN NORMAL SUBJECTS.

Subject	B only	B+0.25T*	B+0.5T*	B+1.0T*	B=2.5T*	B+5T*	B+10T*	B+20T*	B+40T*
d.b.	0	6375	7050	9250	11726	9500	8200	6480	-
$d.b._1$	300	7900	9600	12000	9702	10500	2960	2937	1006
m.m.	0	-	36600	36800	39780	33600	20800	-	-
m.a.	750	3000	7000	6200	6724	6000	4080	3040	-
c.m.	1050	7700	5950	6000	14678	10800	6800	5600	3360
m.f.	100	3000	1050	2950	3813	4750	4360	6976	-
j.d.	2325	28000	27200	32300	32062	11750	17040	10976	3744
n.h.	100	1050	1150	1250	2439	4100	3180	3872	2944
r.y.	0	1900	1800	3875	4387	6300	6740	6080	3424
j.w.	750	6700	7100	5200	4141	10482	6080	2544	1360
p.h.	50	2425	6000	8050	5822	5000	6280	5216	2192
m.e.	0	1550	1530	3075	5526	4300	2560	1568	328
h.k.	700	1050	1525	2600	3198	2912	2110	1200	792
$c.e._1$	575	500	1350	2825	9922	9650	5320	5248	2224
b.t.	150	4200	6850	17300	14678	10400	7280	7232	3208
b.w.	150	1150	1550	1550	1496	2050	1060	968	536
MEAN	438	5100	7708	9764	10634	8882	6578	4662	2093
SEM	±152	±1763	±2503	±2863	±2694	±1831	±1317	±706	±359

(-) = No data. (0) = 0. * = x 10^4 B = 5 x 10^4

TABLE42 - EFFECT ON PFC RESPONSE OF VARYING THE T:B RATIO IN CULTURES CONTAINING B^* + T1250 CELLS IN NORMAL SUBJECTS.

Subject	B only	B+0.25T*	B+0.5T*	B+1.0T*	B+2.5T*	B+5T*	B+10T*	B+20T*	B+40T*
d.b.	-	6050	10750	13000	13776	10937	8980	-	-
$d.b._1$	-	7600	20800	24000	26026	37200	31040	34584	-
m.m.	-	-	20800	39600	32340	16000	15200	-	-
n.a.	-	1700	4250	2050	3854	4250	3880	1584	1016
c.m.	-	12300	12600	14200	10250	9100	10800	8320	7136
m.f.	-	3150	3900	4700	6601	3200	1700	3808	-
j.d.	-	18500	14450	19700	18368	19850	15960	11328	6400
m.l.	-	1250	1150	950	1804	3600	7000	6528	3872
r.y.	-	2000	3650	3925	6806	8975	7580	6832	4334
j.w.	-	4200	3250	6200	10557	17887	7250	5648	2176
p.h.	-	2925	4000	3550	3075	6825	6160	5216	2528
m.e.	-	600	2575	3500	5289	8900	5600	3712	2670
h.k.	-	1150	1900	6200	7790	8000	4880	3152	1544
$c.e._1$	-	1100	1800	3900	4674	6100	3520	4320	2128
b.t.	-	6100	10750	10400	16400	14500	8200	5824	6128
b.w.	-	350	150	200	1558	1712	1620	1328	1536
MEAN	-	4598	7298	9755	10573	11065	8711	7299	3460
SEM	-	±1301	±1707	±2621	±2215	±2198	±1808	±2213	±607

(-) = No data (0) = 0. * = x 10^4 B = 5 x 10^4

TABLE 43 - EFFECT ON PFC RESPONSE OF VARYING THE T:B RATIO IN CULTURES CONTAINING B^{*} + T3200 CELLS IN NORMAL SUBJECTS.

Subject	B only	B+0.25T*	B+0.5T*	B+1.0T*	B+2.5T*	B+5T*	B+10T*	B+20T*	B+40T*
d.b.	-	7100	8625	11750	13530	15100	13820	-	-
$d.b._1$	-	5800	11200	16500	32648	28400	18876	-	-
m.m.	-	-	48000	49200	40656	45000	60480	-	-
m.a.	-	1425	1500	1600	7708	17150	12920	4544	1712
c.m.	-	9800	11500	11600	10906	12250	6800	5120	2720
m.f.	-	1800	5400	7200	8282	6950	3480	2752	-
j.d.	-	16000	19300	17500	14186	19850	15520	15040	7200
n.l.	-	900	1375	1550	2747	8525	9040	7808	4128
r.y.	-	2875	4575	5572	7831	12900	11740	7648	4704
j.w.	-	7625	5450	14350	14309	17087	8270	4752	5568
p.h.	-	2750	3650	4200	4674	8450	6340	5760	4384
m.e.	-	-	-	-	-	-	-	-	-
h.k.	-	1025	2225	5424	6437	9475	6520	5592	1552
$c.e._1$	-	2400	3700	6600	10332	13550	7120	5056	3672
b.t.	-	2900	3750	8300	6437	19250	9920	7136	3248
b.w.	-	600	1400	2450	2214	3850	2800	2432	3840
MEAN	-	4500	8777	10920	12193	15353	12910	6137	3884
SEM	-	±1168	±3075	±3041	±2769	±2617	±3585	±944	±493

(-) = No data. (0) = 0. * = x 10^{4} B = 5 x 10^{4}

TABLE 44- EFFECT ON PFC RESPONSE OF VARYING THE T:B RATIO IN CULTURES CONTAINING B^{*} + T NON IRRADIATED CELLS IN RHEUMATOID SUBJECTS.

Subject	B only	B+0.25T*	B+0.5T*	B+1.0T*	B+2.5T*	B+5T*	B+10T*	B+20T*	B+40T*
M.K.	150	750	1125	3500	2993	1250	2920	2944	1176
V.C.	2500	17000	17000	13400	11480	11800	8240	8128	-
J.G.	0	1300	3800	3000	2132	1538	1070	1024	560
E.L.	200	7800	5000	10400	6200	6600	7760	8778	5610
L.L.	50	3700	1800	800	4774	7500	7280	4092	3234
MEAN	580	6110	5745	6220	5516	5738	5454	4993	2645
SEM	±481	±2993	±2888	±2410	±1649	±1980	±1450	±1499	±1142

(-) = No data (0) = 0. * = x 10^{4}. B = 5 x 10^{4}.

TABLE 45- EFFECT ON PFC RESPONSE OF VARYING THE T:B RATIO IN CULTURES CONTAINING B^{*} + TL250 CELLS IN RHEUMATOID SUBJECTS.

SUBJECT	B only	B+0.25T*	B+0.5T*	B+1.0T*	B+2.5T*	B+5T*	B+10T*	B+20T*	B+40T*
M.K.	-	1000	2850	3525	3259	1850	1060	3520	1176
V.C.	-	18000	20600	21000	16564	8200	9920	5952	2624
J.G.	-	950	500	1725	2562	2000	1750	1488	1376
E.L.	-	7600	10800	10400	13629	14000	35200	33528	-
L.L.	-	200	600	200	1925	6300	6800	19008	8184
MEAN	-	5550	7070	7370	7588	6370	10946	12699	3340
SEM	-	±3389	±3872	±3828	±3108	±2301	±6280	±6038	±1646

(-) = No data (0) = 0. * = $\times 10^{4}$. B = 5×10^{4}.

TABLE 46- EFFECT ON PFC RESPONSE OF VARYING THE T:B RATIO IN CULTURES CONTAINING B^{*} + T3200 CELLS IN RHEUMATOID SUBJECTS.

SUBJECT	B only	B+0.25T*	B+0.5T*	B+1.0T*	B+2.5T*	B+5T*	B+10T*	B+20T*	B+40T*
M.K$_1$.	-	2900	2850	2800	2132	3062	4460	5376	-
V.C.	-	19400	24600	21400	18860	11900	6080	7616	3776
J.G.	-	150	1475	750	-	1125	720	2720	1472
E.L.	-	5800	7000	6000	11088	14500	38000	32800	-
L.L.	-	0	1200	800	3850	5300	8880	24024	10956
MEAN	-	5650	7425	6350	8983	7177	11628	14507	5401
SEM	-	±3597	±4417	±3882	±3822	±2579	±6724	±5895	±2856

(-) = No data (0) = 0. * = x 10^4. B = 5 x 10^4.

responses with T_{1250} and T_{3200} cells occurred at this cell concentration. In 50% of individuals, peak responses for T_{1250} and T_{3200} both occurred at $5x10^4$ cells. A dose response curve could be defined for each individual. Although the peak response occurred at different ratios between individuals, and between T cell types, it was evident that up to an optimal concentration, the addition of more T cells increased the PFC response and that after this point further addition resulted in a reduction. This was true even for T_{3200} cells where it was considered that help was being added in a selective fashion. Finally, in accordance with Thomas et al's[1] observation in 6 out of 16 normal individuals at $0.25x10^4$ and 6 out of 16 normal individuals at $0.5x10^4$, irradiation of T cells at 1250 rads resulted in a decline of the PFC response consistent with a destruction of radiosensitive helper cells.

When the data from control subjects were considered by group (Tables 47-49), it was evident that the peak response (10634 ± 2694) for non-irradiated T cells occurred at $2.5x10^4$ cells, while peak responses to T_{1250} and T_{3200} cells (11065 ± 2198 and 15853 ± 2617 respectively) were coincident at $5x10^4$ (i.e. the 1:1 ratio used in preceding studies). From dose response curves (Figure 40) it was evident, that after these peaks, responses declined with the addition of further T cells. The progressive decline continued even after the $20x10^4$ point

TABLE 47- COMPARISON OF MEAN PFC RESPONSES FROM CULTURES OF B AND NON IRRADIATED T CELLS IN NORMAL CONTROLS AND RHEUMATOIDS.

CELL COMBINATION	PFC/10^6PBL MEAN ± SEM		P
	NORMAL	R.A.	
B only	438 ± 152	580 ± 481	NS
B+0.25T	5100 ± 1763	6100 ± 2993	NS
B+0.5T	7708 ± 2503	5745 ± 2898	NS
B+1.0T	9764 ± 2863	6220 ± 2410	NS
B+2.5T	10634 ± 2694	5516 ± 1649	NS
B + 5T	8882 ± 1831	5738 ± 1980	NS
B + 10T	6578 ± 1317	5454 ± 1450	NS
B + 20T	4662 ± 306	4993 ± 1499	NS
B + 40T	2093 ± 359	2645 ± 1142	NS

TABLE 48- COMPARISON OF MEAN PFC RESPONSES FROM CULTURES OF B AND T1250 CELLS IN NORMAL CONTROLS AND RHEUMATOLDS

CELL COMBINATION	PFC/10^6PBL MEAN ± SEM		
	NORMAL	R.A.	P
B + 0.25T	4598 ± 1301	5550 ± 3389	NS
B + 0.5T	7298 ± 1707	7070 ± 3872	NS
B + 1.0T	9755 ± 2621	7370 ± 3828	NS
B + 2.5T	10573 ± 2215	7588 ± 3108	NS
B + 5T	11065 ± 2198	6370 ± 2301	NS
B + 10T	8711 ± 1808	10946 ± 6280	NS
B + 20T	7299 ± 2213	12699 ± 6038	NS
B + 40T	3460 ± 607	3340 ± 1646	NS

TABLE 49 COMPARISON OF MEAN PFC RESPONSES FROM CULTURES OF B AND T3200 CELLS IN NORMAL CONTROLS AND RHEUMATOIDS

CELL COMBINATIONS	PFC/10^6PBL MEAN ± SEM		P
	NORMAL	R.A.	
B + 0.25T	4500 ± 1168	5650 ± 3597	NS
B + 0.5T	8777 ± 3075	7425 ± 4417	NS
B + 1.0T	10920 ± 3041	6350 ± 3882	NS
B + 2.5T	12193 ± 2769	8983 ± 3822	NS
B + 5T	15853 ± 2617	7177 ± 2579	< 0.05
B + 10T	12910 ± 3585	11628 ± 6724	NS
B + 20T	6137 ± 944	14507 ± 5895	< 0.025
B + 40T	3884 ± 493	5401 ± 2856	NS

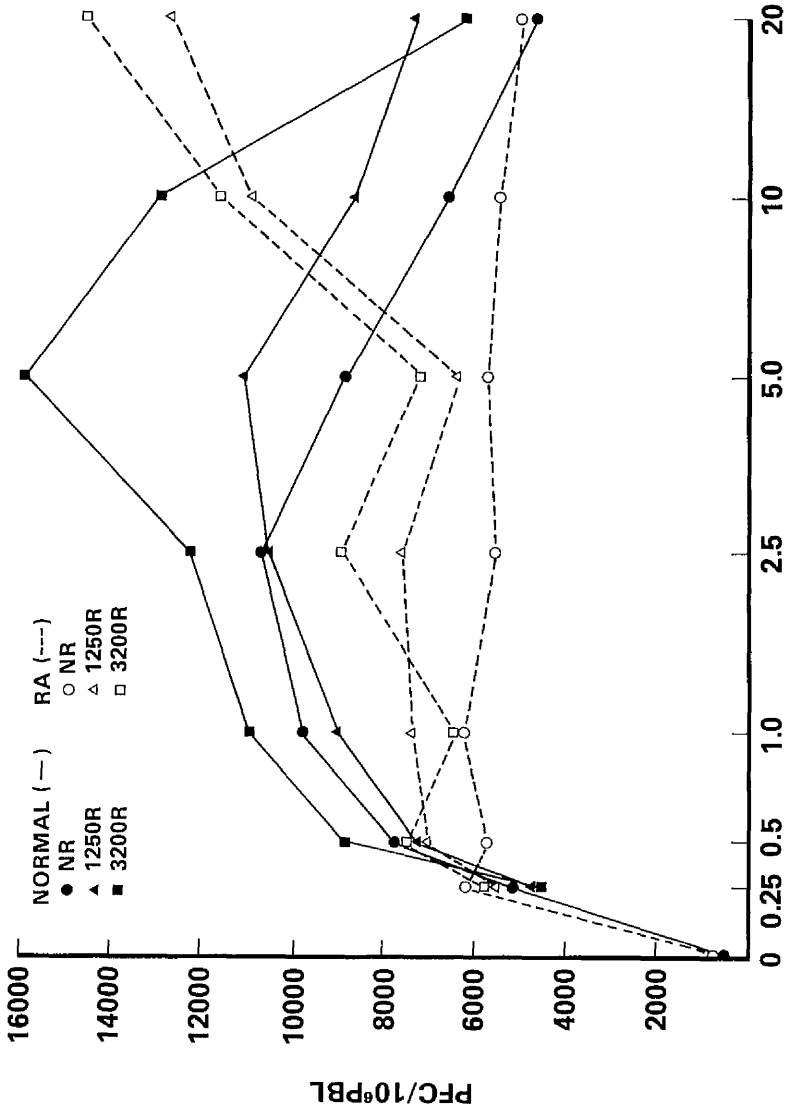


FIGURE 40: DOSE-RESPONSE CURVES OF MEAN PFC RESPONSES FOR RHEUMATOIDS AND NORMAL SUBJECTS AT SELECTED T/B RATIOS.

displayed in Figure 40 (see Tables 47-49). Adding even a small number of T cells ($0.25x10^4$) resulted in a significant augmentation of the PFC response (438 $\pm$ 152 to 5100 $\pm$ 1763 for non irradiated T cells). While not being statistically significant ($p>0.05$), in the group analysis it was noted that at T cell concentrations below $1x10^4$, (i.e. low T/B ratios) low dose radiation resulted in a decline in immunoglobulin synthesis (5100 to 4598 at $0.25x10^4$ and 7708 to 7298 at 0.5×10^4 T) while at higher T/B ratios (greater than $2.5x10^4$ T cells) the same dose of radiation resulted in enhancement (8882 to 11065 at $5x10^4$ T). Maximum radiation enhancement ($B+T_{3200}$ + PWM / B+T+PWM) occurred at a 1:1 ratio (i.e. $5x10^4$ T cells) as noted in previous experiments.

When rheumatoid data were considered (Tables 44-46), certain features of the response were similar. Peak responses for given combinations (B+T, $B+T_{1250}$ and $B+T_{3200}$) occurred at different T/B ratios for different individuals, the variation being greater than that observed in controls. There was significant individual variation in PFC response at any defined T/B ratio of a given cell combination (e.g. $B+5x10^4T_{1250}$). Peak responses for non-irradiated T cells, T_{1250} and T_{3200} cells for the same individual were not coincident. While the addition of non irradiated T cells significantly enhanced the PFC response above that from B cells cultured alone (580 to 6110, 5550 and 5650), dose response curves were flatter than in controls (Figure 40).

It was evident from the group data (Tables 46-48) that the peak response (14507 $\pm$ 5895) occurred with T_{3200} cells at a high T/B ratio ($20x10^4$ T cells), a feature not seen in control cultures. Thus the normal dose response profile with a peak at a 1:1 T/B ratio was absent. In further considering these data by group (Figure 40) certain differences between rheumatoids and controls were noted. Below 10^5 T cells responses in rheumatoids were lower than in controls, dose response curves being flat for non-irradiated and irradiated T cells. At a 1:1 ratio the failure of significant radiation enhancement noted was consistent with that observed in previously described studies and contrasted the substantial enhancement seen in normals.

With the addition of more non-irradiated T cells the response in rheumatoids showed only a slight decline (cf normal subject). However, when more irradiated T cells (T_{1250} or T_{3200}) were added, the response progressively increased ($B+T_{3200}+PWM$ greater than $B+T_{1250}+PWM$) and at $20x10^4$ cells radiation enhancement was similar to that seen in controls (but at $5x10^4$ cells). It was evident from these observations, that large numbers of T_{1250} or T_{3200} cells which would have suppressed the PFC response in normals were capable of enhancing the response in rheumatoids. Furthermore, while significant radiation enhancement was not evident with $5x10^4$ cells, a normal degree of enhancement could be achieved with the same degree of radiation but larger numbers of T cells. The peak of this dose response curve for rheumatoids

was between $20x10^4$ and $40x10^4$ T cells (Tables 47-49) since responses declined between these two points (T non irradiated 4993 to 2645, T_{1250} 12699 to 3340 and T_{3200} 14507 to 5401).

When group means were subjected to statistical analysis using students t test (Tables 47-49) a statistically significant between-group difference was found at only two points, B+$5x10^4$ T_{3200} and B+$20x10^4$ T_{3200} (Table 49). These points were coincident with those at which maximum radiation enhancement occurred in normal controls and rheumatoids respectively.

Finally, in support of statements made in Chapter 4 regarding possible drug effects, it was noted in this study that normal subject hk was on Motrin 1600 mg/day for osteoarthritis, but nevertheless radiation enhancement at a 1:1 T/B ratio was normal (2192 to 9475).

DISCUSSION:

In this study the reverse hemolytic plaque forming cell assay was used to further dissect complex T-T and T-B cell interactions, which are important in the induction and homeostatic regulation of human B cell differentiation triggered by pokeweed mitogen. Four important observations were confirmed in normal individuals:

1. The addition of relatively small numbers of T cells had a profound effect on the B cell response.

2. The suppressive effects of radiation (1250 rads) at low T/B ratios and the enhancing effects at high T/B ratios were confirmed, although suppressive effects were not dramatic. This was consistent with the existence of a radiosensitive subset of T helper cells. With higher doses of radiation (3200 rads) in 1:1 ratio combinations the effect was always to enhance the PFC response, suggesting that this dose destroyed more radiosensitive T suppression than radio-sensitive T help. It was not known whether radiosensitive and radioresistant helper T cells were distinct subsets within the OKT4+ population.

3. Peak radiation enhancement was noted with 3200 rads in 1:1 ratio B/T combinations confirming that these previously chosen conditions were optimal for

observing maximal effects. However, the individual variation noted in both rheumatoids and normals indicated that large numbers of subjects were necessary to detect between-group differences.

4. It was evident that the resppnse to T cells was not a linear increase in the PFC response. Although results obtained at high T/B ratios may be complicated in part by non-specific effects of cell cultures, e.g. nutrient competition, contamination with macrophages or other cells, it has also been noted that cells respond to crowding and also that an excess of helper signals may have the effect of suppressing the B cell response. It has been demonstrated in this system that the decline in response cannot be explained on the basis of B cell exhaustion. An alternative explanation suggested by Thomas et al[363] is that OKT4+ cells may be capable of generating suppressor factors independent of OKT8+ cells and therefore as more helper (OKT4+ or radioresistant) cells are added, the result may be the generation of more suppressor factors within this population.

Recent observations[373] have suggested four possible ways of explaining immunoregulatory phenomenon.

(a) The radiosensitive OKT4+ population contains not only cells important in inducing B cell differentiation but also cells intimately involved in suppression.

(b) The target of suppression by OKT8+ cells is the radiosensitive OKT4+ cell.

(c) The OKT4+ population contains precursor cells which can be induced to differentiate into mature helper or suppressor cells.

(d) A radiosensitive cell within the OKT4+ population may be programmed for help or suppression.

Thus it appears likely that further heterogeneity within the OKT4+ populations will be detected.

While these techniques have previously been applied in normal subjects there are no prior reports of their systematic application in the study of patients with rheumatoid arthritis. It was clear from the data that striking differences existed between the controls and rheumatoids. Of greatest importance, was the differential effect which increasing numbers of irradiated T cells had on rheumatoid versus normal B cells. While significant radiation enhancement could not be observed in rheumatoids at a 1:1 ratio it was entirely normal at a 4:1 ratio. This reinforced the point that given appropriate helper signals, B cells could respond normally in this system and therefore that the restricted PFC response seen in rheumatoid arthritis was not a consequence of hyporesponsiveness of B lymphocytes. To achieve the maximal

response, 4 times as many irradiated R.A. T cells had to be added supporting the existence of restricted T helper activity in rheumatoid arthritis. Of interest, increasing cell density did not appear to have the same inhibitory effect on rheumatoid cultures, as was seen in normals (i.e. a decline in the PFC response between $5x10^4$ and $40x10^4$ T cells in normal subjects and between $20x10^4$ and $40x10^4$ in rheumatoids), suggesting perhaps that either the ability to sense overcrowding was impaired, or that these hypofunctioning cells did not utilize culture nutrient as rapidly as normal cells, or that suppressor factors were not released in rheumatoid cultures as they were in control cultures. Finally, the importance of standardization of the procedure was illustrated. If $20x10^4$ T to $5x10^4$ B cells had been chosen as the working ratio, radiation enhancement would have been observed in rheumatoids but not in normal subjects, and if 10^5 T cells to $5x10^4$ B cells had been chosen no difference would have been observed.

These experiments demonstrated that the rheumatoid B cell had the potential for normal immunoglobulin synthesis and that T helper function was restricted. Whether this restriction was primary or secondary to the disease was not known. It is possible that T helper function was preoccupied, i.e. because of prior commitment in vivo, T cells were not capable of further in vitro response. Evidence cited suggests that suppression is mediated

through T helper cells. Since in this assay it is only possible to appreciate suppression in the presence of normal help and since help was shown to be deficient, little could be said regarding the normality of suppressor activity. While OKT5+ and Tγ enumeration studies indicated that the number of suppressor cells was normal their function remained uncertain. In the final discussion these observations will be viewed in the light of available evidence to prpvide some understanding of regulatory events detected by this assay.

SUMMARY:

Five patients with rheumatoid arthritis and sixteen normal subjects were evaluated using a modification of a previously described method. Although purified T cell subsets were not used because of the high cost of monoclonal antibody, observations in normal subjects were consistent with those achieved using purified subsets. It was evident that only a small number of T cells were required to augment the PFC response from B cells as measured by the reverse hemolytic plaque assay. Up to a certain point the response could be increased by adding larger numbers of T cells, after which there was a progressive decline in the response. While in the past this response has been attributed to non-specific effects of high density cell cultures more recent evidence suggests that this response may be due to the release of suppressor signals as a consequence of overcrowding, or of the generation of suppressor

cells from the OKT4+ population. The data were consistent with the existence of a radiosensitive population of helper T cells, although this effect was not as marked as reported by other researchers using purified T cell subsets. While these techniques have been applied to the study of normal subjects, there is no evidence of their having been applied systematically in the study of patients with rheumatoid arthritis. Consistent with conclusions drawn in preceding chapters the B cell was shown capable of responding normally to appropriate helper signals while helper T cell function was shown deficient. While the PFC response could not be enhanced in a normal fashion by the addition of non-irradiated T cells, the addition of large numbers of irradiated (T_{1250} and T_{3200}) cells produced PFC responses and radiation enhancements similar to those seen in normals. Finally, the inhibitory effect of progressively increasing T cell numbers (as seen in controls above $5x10^4$ T cells) was only observed at higher cell concentrations in rheumatoids, suggesting that apart from larger numbers of T_{3200} cells contributing more help that the regulatory signals of overcrowding or suppressive effects of excessive help were not evident in rheumatoid cultures, (at equivalent T/B ratios).

CHAPTER 12

FINAL DISCUSSION AND CONCLUSIONS

Is obair-la toiseachadh - gnath - fhacal

Ach is obair beatha criochnachadh - beachd
a chomh chruinniche.

Translation.

Beginning is a day's work

Gaelic proverb

But finishing is the work of a lifetime.

Authors note.

In spite of intensive research over the last several decades the disease rheumatoid arthritis remains one of modern medicines greatest enigma's. In Chapter 1, evidence favouring recent evolution of the disease was discussed. While the absence of finding definite evidence of rheumatoid arthritis has been used to infer that it was absent prior to fairly recent times, this can only be regarded as indirect proof. It may have been considered unacceptable to paint crippling deformities during the Middle Ages explaining the apparent lack of pictorial evidence. While a fused bony ankylosing spondylitic spine may withstand centuries of burial, the more delicate soft tissues and cartilaginous changes of the rheumatoid could be expected to decay, possibly explaining the difference between historical evidence for ankylosing spondylitis versus rheumatoid arthritis. The notable archeologic finds have been in the tombs of the nobility, who represented only a very small percentage of the population of interest. If Sydenham was correct[12] and the aesthenic gout "lived in the houses of the poor", then it is not surprising that rheumatoid arthritis has been infrequently described, since much of this work was conducted in the well preserved bodies of the rich.

The possible scarcity of rheumatoid arthritis in early history is in sharp contrast to the frequency and ubiquity with which it has been noted in the 20th Century[24]. This change in prevalence may reflect more ready recognition of the disease by

physician and lay person, its recent emergence possibly due to the evolution of a new microbe, reclassification of various forms of arthritis or an increase in frequency of a previously rare but existent disease possibly due to environmental factors of industrial society, and/or a change in host susceptibility, or that due to increased longevity more people survive long enough to develop the disease. There has been some speculation regarding the possibility of a decline in the frequency and severity of rheumatoid arthritis in recent years[366, 367]. While the evidence is weak it has been the impression of some experienced rheumatologists that they are not seeing the severe forms of rheumatoid vasculitis or as many severe forms of rheumatoid arthritis as in the 1950-1960's. The increased frequency of vasculitis in previous decades, has been attributed to the more frequent usage of high doses of steroids, although evidence to support this hypothesis is weak. The decrease in the frequency of severe forms of arthritis has been variably attributed to a change in the natural history of the disease or the effects of improved forms of therapy. If indeed, as seems likely, that rheumatoid arthritis is virally triggered then there is precedent in the infectious disease literature for a rise and fall in the frequency of disease. Typhus, tuberculosis and leprosy were formerly more common than they are now. Examination of the evidence pertaining to the decline in tuberculosis indicates that it was already falling in frequency

prior to the introduction of specific treatment and public health measures. Since the accurate recording of disease frequency is complicated by the difficulty of correctly identifying all those who do or do not have the disease at a given point in time, and since these types of record have only been compiled in the last several decades it is hardly surprising that it is difficult to detect changes in frequency or severity in a disease which is possibly changing very slowly. Since treatment modifies disease activity and severity, any improvement in this direction may be wholly attributed to therapy rather than to a change in natural history. However, there is no form of therapy known to prevent the development of rheumatoid arthritis. Studies on disease frequency may provide useful information, provided the sensitivity and specificity of diagnostic criteria do not change.

The histology of rheumatoid inflammation has been well described, particularly synovial pathology, where tissue is easily and frequently obtained. The vasculitic lesions of the digits and the scleral lesions of the eye represent the most severe extra-articular manifestations of the disease, which fortunately are infrequent. Some controversy surrounds the origin of the rheumatoid nodule. While its characteristic three layer structure of central necrosis surrounded by a palisade of histiocytes and an outer layer of lymphocytes and plasma cells has been well defined, evidence supporting the contention of some pathologists that this represents

a response to previous vasculitis is inconclusive. The morbidity associated with a destructuve synovitis or severe vasculitis, the high frequency and ubiquity of rheumatoid arthritis, the high cost of managing patients with the disease, and the extremely high economic cost in terms of lost productivity[186, 187], highlight the importance of this disease in 20th Century society.

The history of immunology represents one of the most exciting developments of the last century. Advances in technology combined with the power of the human intellect have allowed progressively greater insight into the complexity of the immune response. The development of the microscope was vital to the realization that different types of white corpuscles existed and that some of these were phagocytic. While this phagocytic activity was for some time considered a basic biologic response to microbes, the recognition of Ia antigens on monocytes and Fc and complement receptors on both monocytes and polymorphs, have suggested a more specific mode of action for these cells. While the cellular and humoral schools of the late 19th Century both contributed to the definition of the immune response, Ehrlich laid the ground for further progress when he recognized the existence of different types of white blood cell. Although several decades have passed, we continue to subdivide even those groups, a process which is not yet complete. It is this dissection of populations of immunocompetent cells which has allowed us to appreciate the complexity of the immune response and

the way it is regulated. Burnett established the concept of a differentiated immune system programmed for a variety of specific responses - a principle which has been carried to succeedingly higher levels of sophistication by subsequent researchers. While methodology still restricts complete appreciation, it has been possible in the past ten years to partially dissect the immune response and appreciate that most important mechanism whereby the response is regulated. While other cell subsets may be further subdivided, current efforts to evaluate the functional activity of subsets of T lymphocytes has been most rewarding. Furthermore, evidence suggests that a cell, at one time thought to be just a scavenger (the monocyte), is not only immunocompetent in its own right but also plays very important roles in regulating other cells, determining the specificity of the immune response, conferring immune maturity on T cells, and is also capable of angiogenesis and tumour surveillance. Research into the activities of this cell promise to provide essential information necessary for further progress in this field. The problems of studying the complex problem of immunoregulation will be discussed later.

Chapter 2 reviewed the immunopathogenesis of rheumatoid arthritis. There is no question that the immune response is stimulated in rheumatoid arthritis and that regulatory function has failed, the majority of the evidence coming from in vitro observations. It is apparent that the immune response is directed

in a number of different directions, some seemingly primary responses, while others are secondary. A foreign antigen has not been detected although it seems likely that this is the trigger. Recent studies with Epstein Barr virus[38] in selected cell lines indicate that the incorporation of viral DNA into the host cell nucleus has the potential to produce established cell lines which perpetuate an aberrant immune response. Furthermore, a number of viruses appear capable of this activity. Some of the immune complexes detected in the sera and synovial fluids of patients with rheumatoid arthritis may indeed contain these foreign antigens. However, rheumatoid factors (i.e. immunoglobulins directed against Fc fragments of IgG) of varying specificities and the production of self-associating IgG by synovial cells[141] indicate that immune complexes can be formed entirely between immune-products of the host. It is thought that this occurs because an extrinsic agent alters self-determinants and renders them antigenic, or are so similar that cross reactivity occurs. Nevertheless, these complexes in the absence of demonstrable foreign antigens appear to play a role in the immunopathogenesis of rheumatoid arthritis. One of the consequences of ongoing inflammation is the destruction of collagen. Altered collagen is antigenic and therefore the host mounts an immune response and produces anticollagen antibodies. These events are most likely secondary and may be important in perpetuation of the disease. Antibodies to nuclear factors (ANA) may be the

result of viral alteration of host nuclear components and be primary, or they may be the result of cell destruction by inflammatory mediators and therefore be secondary. Finally, immune complexes may be laid down in the interstices of degraded cartilage[150-152]. While they cannot be engulfed by phagocytosis (frustrated phagocytosis), they provide a constant source of immune stimulation which results in perpetuation of the release of biologically active molecules[155, 156] as a consequence of attempted phagocytosis, which results in further tissue damage. Perpetuation is mediated then, not only by cellular activity but also by the release of lymphokines and monokines.

Immune complex formation leads to the effector sequence mediated by both cellular and humoral instruments. Complement activation results in cell lysis but also in the ingress of phagocytic cells into the area of inflammation. The consumption of immune complexes by polymorphs and of antigen by monocytes results in the release of many biologically active molecules, some of which, prostaglandins, superoxide radicals, collagenases, elastases and cathepsins have the effect of damaging host tissue producing periarticular osteoporosis, erosions, bone cysts and the destruction of cartilage. These events in turn release altered host proteins which are themselves antigenic.

While in vitro observations together with this construct explain in part the histologic and immunologic abnormalities in rheumatoid joints, too little is known regarding the earlier phase

of the response and its regulation. Since the immunocompetent cells which are recruited into the joint are derived from circulating leukocytes, the peripheral blood is a useful site from which to easily obtain cells and examine any abnormalities which may exist. The presence of circulating immune complexes in peripheral blood and the development of multiple extra-articular lesions suggest that these abnormalities are not confined within the joint capsule.

During the two years in which these studies were conducted, new methodologies were developed in several countries. While these techniques had been evaluated in normal subjects, for some it marked the first time in which they had been applied in the systematic study of patients with rheumatoid arthritis. Since these techniques had not been widely applied it was necessary not only to define normality by using a control group of normal subjects, but also to study sufficient patients with disease to allow detection of differences. Furthermore, many methods were technically difficult and required considerable learning experience before studies could be conducted with confidence. The adaptation of methods reported by other researchers using different systems, required verification and standardization prior to application.

Some of the problems encountered during the two years will be presented in order to demonstrate some of the difficulties in

conducting this type of research. Problems arose at a variety of levels and involved both materials and techniques, however problems with materials were reasonably limited. An infrequent but recurrent problem was the cracking of plastic centrifuge tubes. In spite of careful visual checking, it appeared that some of these tubes cracked in the centrifuge resulting in the loss of all or most of the contents. On one occasion the facilitating serum used for plaque incubation became contaminated resulting in complete absence of the PFC response and the discarding of all results. The majority of problems arose as a consequence of biological or technical variation.

Problems of a methodologic nature were more frequent and are best presented in the order in which they were encountered. The density of Ficoll Hypaque was critical, deviation from the optimal being associated with an increase in contamination with granulocytes. It was noted that not all patients "Ficolled" well. Instead of forming well defined layers, there was contamination of the lymphocyte layer by red cells and either clumps of white cells or fibrin. This was infrequent, tended to occur to different extents in tubes from the same patient and did not apparently compromise the study endpoints. The preparation of nylon wool columns required that a thin strand of nylon wool be packed evenly inside a bevelled bottom drinking straw, and that it was not unduly compressed in any area or compressed into the

bottom 2-3cms of the tube. Several tubes were discarded after preparation, either because airlocks formed or because compressed areas became evident and did not absorb the foetal calf serum, or because the hole created in the bottom of the tube was too large or too small. It was more efficient to make a batch of columns rather than just prepare the three to four needed on each occasion. Meticulous attention was paid to suspending cells prior to loading on to the column. Suspension was achieved using a Pastour pipette, a process which tended to produce bubbling. It was important that the pipette bulb be carefully controlled so that (a) cells were not left in the froth at the bottom of the tube and (b) that air bubbles were not introduced into the column since this prohibited the addition of foetal calf serum in RPMI 1640 to the top of the column and resulted in drying during the incubation. A second technical problem associated with separation, was controlling the force applied when massaging the tube to detach adherent cells. Too little pressure resulted in the retention of cells on the column, while too vigorous massaging traumatized the cells reducing cell viability and compromising performance. However, with experience results were consistent and cell viability and performance maintained.

When cells were irradiated prior to coculture, the duration of exposure (seconds) needed in the Gammacell to give a set dose of radiation gradually increased, due to decay of the isotope. It was necessary therefore to recalculate the exposure time periodically.

Bacterial contamination of cultures was an ever present problem. With careful attention to the way in which cell suspensions, reagents and pipettes were handled and with close monitoring of the cleanliness of the incubator, contamination was reduced to less than 1 in 200 cultures. Contaminated cultures were discarded as soon as possible to avoid any spreading of infection.

The coupling of polyspecific antisera to sheep red blood cells with aged chromium chloride is a delicate and critical process which was suspected as being the cause of total failure of PFC responses on some days. It is important to assess the health of sheep red blood cells prior to usage. If the blood is old it usually appears darker and the cells are morphologically abnormal, often being crenated. Only healthy cells should be used. The age of the chromium chloride is important, failure having been reported with the use of fresh reagent. It is necessary to prepare sufficient quantities initially to replenish the supply as necessary, since ageing takes several months. If these two conditions are assured then the final problem arises with coupling the antisera. On occasions, and for no apparent reason this failed. Because coupling failure results in the total loss of PFC response and therefore the loss of valuable data, coupling was verified with human Cohn fraction II in an agglutination reaction. If no plaques are obtained in any culture combination, then it is most likely that coupling is the source of the problem. If cells have not been discarded then

it is possible to salvage the experiment by coupling a new batch of cells and repeating the procedure again on the same day. The known decline in PFC response with longer periods of culture prohibits holding cells over until the next day.

The predominant problem in the last half of the assay related to the counting of plaques. The desirability of culturing, that number of cells which would produce an accurately countable number of plaques, was previously mentioned. The execution of a study on four individuals consumes the most part of a day and early evening. If the response fails then cells have to be re-plaqued with newly coupled cells then there is insufficient time to count the more than 100 slides produced. In these instances counting may be left until the next morning providing that slides are refrigerated overnight. In order to determine whether this delay introduced any inaccuracy some samples were counted on the same day as the study and again after overnight refrigeration. It was evident that no change occurred providing that storage conditions were adequate and slides were not allowed to dry out.

It is evident that the magnitude of the PFC response is related to the source and batch of pokeweed mitogen, foetal calf serum, polyspecific antisera and complement, and therefore it is important to (a) standardize the method, (b) utilize a constant source of batch of histological reagents, (c) restandardize when source or batch change and (d) not combine results obtained from experiments utilizing different biologic materials. Results varied between cultures and between plaques obtained from identical

cell suspensions necessitating the utilization of replicate determinations.

The Tγ enumeration and serial depletion experiments were relatively problem free, although it was necessary to check new batches of chicken red blood cells to ensure that cells were in a healthy condition. Coupling Ig anti-CRBC to CRBC was invariably successful. Endpoints could be easily read. It has been suggested that T cells bearing Fc receptors for IgM (Tμ) are helper T cells. Attempts were made to enumerate Tμ cells by a similar method to that used for Tγ enumeration, but differing in the class of antiserum and the type of red cells used. The rosettes, which have been noted by others using ox red blood cells coated with anti IgM antiserum, could not be duplicated. It was suspected that the ox blood was not in good condition on arrival, although Tμ rosetting has not generally been as technically adequate as Tγ rosetting. No results were obtained from these experiments and therefore the details of the method have not been reported.

The problem of enumerating monocytes by utilization of their ability to phagocytose latex particles[296] was previously alluded to. In order to be accurate it appears that (a) the minimum number of particles necessary to detect the maximum number of monocytes should be used and (b) that cells require to be washed many times in order to remove any non-phagocytosed latex. Only in this way can the

inaccuracy inherent in counting cells to which latex beads have adhered (and appear by superimposition to have been phagocytosed) be avoided.

The techniques used to conduct Ia, OKT4 and OKT5 enumeration studies were similar. As with all experiments it was necessary to ensure adequate resuspension of cells prior to introduction into either culture tube, plaque assay or microcytotoxicity assay to avoid pipetting from partially precipitated cell-rich areas at the bottom of the tubes or the cell-poor area at the top of the tube in suspensions that had been standing. The most important details of these microcytotoxicity assays were the precision of introduction of cells and reagents into each well, and the necessity for complete mixing. It was found that cytotoxicity counts 24 hours apart were the same, indicating that it was not necessary to count percent kill on the same day as the experiment (preparations should however be kept refrigerated). One preparation was refrigerated for four months and remained in remarkably good condition, suggesting that once stained and fixed the preparation had considerable longevity.

Monocyte enumeration by the nonspecific esterase method necessitated certain decisions regarding which cells to count. Granulocytes were recognized by their uptake of methyl green and their nuclear morphology. They were relatively infrequent and

and were not counted. Isolated lymphocytes (green) and monocytes (brown/red) were also identified by their colour, size and morphology. Two further situations arose. In the first cells were present which failed to take up any stain. They were either red cells or effete leukocytes and so were not counted. The more difficult decision was whether monocytes that had formed clumps with other monocytes should be counted. This phenomenon only occurred in some areas of some preparations and was not different in frequency or degree between rheumatoids and controls. Since there were invariably other areas of the preparation in which clumping was absent and monocyte/leukocyte distribution more even, these areas were avoided. This feature requires recognition since high monocyte/lymphocyte ratios in these areas have the potential for distorting the result. Also because distribution is not entirely uniform it is necessary to count large numbers of cells.

Studies on allogeneic cultures, autologous factors and varying B/T ratios were conducted in a methodologically similar manner to studies 1-6. The problems encountered did not differ between these studies. Since the number of B cells obtained from any given patient was less than the number of T cells, it was this former subset of lymphocytes which limited the number of cultures or culture combinations which could be established from a given volume of peripheral blood.

Reliability and validity are two attributes of a procedure, the first of which describes the consistency or reproducability while the latter refers to the accuracy, i.e. does it measure what it is supposed to. When the same cells were cultured in replicate and each replicate plaqued once, or when cells from a single culture were plaqued in replicate, the PFC responses recorded were not identical. While the magnitude of the differences between rheumatoids and normals in radiation experiments was much greater than this, nevertheless this small variability in the procedure should be noted since small differences previously alluded to may be within the variability of the test. Furthermore, it illustrates the necessity of conducting replicate determinations in this type of work rather than relying on a single figure for comparison.

There is reasonable evidence (Chapter 3) to indicate that the RHPFC assay is accurate, that is to say, it does measure immunoglobulin synthesis in vitro. The method has significant advantage over several other methods in that it measures cells synthesising immunoglobulin at a point in time, rather than the result of the production of preformed immunoglobulin and the sum of several days immunoglobulin synthesis in vitro. Thus, it is able to assess the function of individual B lymphocytes at a point in time. While early work suggested that relative radiosensitivity could effectively separate functional subsets of T cells, it is clear that the effect of radiation is more complex. Both the dose of radiation

and the cell concentration appear to have a bearing on the outcome observed. While suppressor T cells are maximally sensitive to an intermediate dose of irradiation, it is clear from dose response curves that the help suppression balance is altered by even lower doses of irradiation, indicating that a spectrum of radiosensitivity exists for suppressor T cells. In respect of helper T cells a broad spectrum of radioresistance can be defined, which is at least partly dependent on the cell concentration[363]. Thus at one end of the spectrum there exist a population which are radiosensitive at 1250 rads, while at the other end there exists a group of cells which are radiosensitive at 3200--6400 rads and a smaller population which are radioresistant at still higher dosages since a return to the level of PFC response observed in cultures containing B cells only is not quite achieved at 6400 rads. Therefore, it can be appreciated that the observed effect is the net result of damage inflicted on radiosensitive suppressor T cells and radiosensitive helper T cells. Again because of this variability in the response, conditions must be clearly stated, controls established and experiments conducted in replicate.

Although it has been assumed that radiation has an effect on helper and suppressor T cells, any differential effect on null cells or monocytes has not been accounted for. Since the proportion of these cells, particularly the latter, is small in T cell preparations, it is considered that any effect were it to exist,

would be negligible. One final comment in this respect, is the observation by some researchers [266, 267, 268] that some T cells adhere to the nylon wool column and that their activity is one of suppression. While columns were not examined to determine what cells were still adherent, after completion of separation it could be said that:

a) Cell yields in rheumatoids and controls were similar.

b) Cell viability after separation was similar in the two groups (99-100%).

c) Controls and rheumatoids were evaluated on the same day using identical techniques.

d) PFC responses from cultures of B cells only and T cells only indicated clean separation.

e) Results from cultures of B + nonirradiated T cells (1:1 ratio) were similar in the two groups.

Taken together these factors indicate that nylon wool separation by itself did not determine the differences observed between the two groups. Since responses from unseparated lymphocytes (cells which were not separated on nylon wool columns) were similar in both groups, it can also be said that Ficoll Hypaque centrifugation did not produce an in vitro artefact.

The data on Tγ cells have to be considered in the light of certain information. Recent evidence has suggested that the original concept of an Fc receptor for IgG being a specific cell membrane marker for a suppressor T cell is not entirely correct. It now appears that not all suppressor cells are Tγ cells (or indeed T cells) and that not all Tγ cells are suppressor cells (or indeed T cells)[285]. Furthermore, those T cells which are Tγ cells seem to have the potential to transform into Tμ cells[289]. These observations have been made in different and not strictly equivalent in vitro systems. While this lack of methodologic sophistication may be disconcerting, nevertheless the technique described in Chapter 5 was highly reproducible and was capable of identifying large differences between rheumatoids and normals, which were consistent with similar differences demonstrated by other techniques. The observation that radiation of T nonγ cells could not further enhance the response, suggests that the radiosensitive suppressor T cell is contained within the Tγ fraction. This is consistent with the predictive effect that removal of T suppressor cells by Tγ rosetting or destruction by optimal dose radiation has on the PFC response of normal subjects.

Enumeration of monocytes by the non-specific esterase method employing pararasanoline and methyl green stains was an effective and efficient method[297]. Although non-specific esterase dots have been noted in T cells (and illustrated in Chapter 6) there is no good evidence indicating that they are a marker for

a pure functional subset of T cells. Their true significance remains to be determined.

The recognition of Ia positivity has been an important link between immunology and genetics. Since non-T cells express Ia antigens it is important to be certain as to which cell the Ia status is being applied. Evidence suggests that Ia positivity is not a specific marker for a helper cell but rather that it is an expression of the activation status of a cell (helper or otherwise). Thus with stimulation, percent Ia positivity increases but a maximal response can only be achieved by the cooperation of OKT4+ Ia- and OKT4+ Ia+ cells. Since some helper T cells may be Ia-, some Ia+ and some produce Ia+ soluble factors, it seems likely that these immunogenetic molecules may exist within the cell cytoplasm, on the cell membrane or be secreted into the surrounding milieu, a feature which may allow a specific response to be mediated in several ways.

While the advent of monoclonal antibodies promises to provide a method with the necessary sophistication with which to make substantial progress, it should be appreciated that not all commercially available monoclonal antibodies are of equal purity or avidity. Although not incriminating the OKT4 and OKT5 antibody used in these studies, a recent study suggested that some of the cells being identified by another monoclonal antibody (OKM1) were not cells for which it was claimed the antibody was specific (monocytes). Furthermore, even with highly purified antibody,

responses are dependent on the laboratory procedure and the indicator technique used. It is likely that the current microcytotoxicity assay will be succeeded by the use of the immunofluorescent cell sorter. In this latter method following incubation of cells with monoclonal antibody a fluorescent label is applied and the cell suspension sorted by a laser beam into fluorescent and non fluorescent fractions. The accuracy of this method is better than that of microcytotoxicity and sterile preparations can be obtained which may then be used in functional studies. In order to determine endpoints in the system used in Chapter 8 it is necessary to stain and fix cells under oil, such that they are unusable thereafter. Nevertheless, with this technique and a source of superior monoclonal antibody it was possible to describe ranges of values in normals which were similar to those described by others, and to describe a range of values in rheumatoids which had not previously been defined at the time of study.

Having outlined the limitations of techniques used, I will describe the inferences which may be made regarding the immune response and its regulation in normal subjects, and then indicate how these studies contribute to the present state of knowledge regarding the same variables in subjects with rheumatoid arthritis.

Studies 1-6 illustrate that in normal subjects immunoglobulin synthesis by B cells can be augmented several fold by coculture with T cells, indicating a net helper activity for this

subpopulation of lymphocytes. That this is a "net" effect can be appreciated by the alterations in response which occur with manipulation of the T cell population. Thus at 1250 rads (and low T/B ratios) the balance is shifted in favour of suppression by the preferential destruction of radiosensitive helper T cells. At 3200 rads the balance shifts in favour of help as radiosensitive suppressor T cells are functionally destroyed with greater effect than either the radiosensitive or radioresistant helper T cells. This shift in balance is recognized by an increase in the PFC response at certain doses of radiation. At even higher doses of radiation (6400 rads) the balance shifts again, as radioresistant helper T cells are destroyed and the response declines towards that achieved by B cells alone. Since a return to baseline is incomplete it can be concluded, as mentioned previously, that at least some helper T cells are resistant to this dose of radiation, although they may be destroyed by even higher doses. Whether radioresistant suppressor cells exist cannot be concluded from this study, but if they exist they are in a minority and their effect is exceeded by that of the radioresistant T helper population. Thus, while a given dose of radiation may not be specific for a particular and pure functional subset of lymphocytes, it is clear that this technique can effect shifts in the net activity of the help/suppression balance. Comparison of PWM^{+} and PWM^{-} cultures,

suggests that in response to a mitogen the magnitude of the shifts in the help/suppression balance can be augmented as a consequence of increased helper T cell activity i.e. peak PFC responses are higher and the post peak decline steeper.

In an unregulated immune response one can conceive of initial mitogen/antigen processing by a monocyte, followed by presentation to a helper T cell, which in turn activates a B lymphocyte resulting in antibody production against the initial antigen. Without modulation these events could result in the excessive and continued production of antibody, since once activated, helper T cells would continue to react with B cells in the absence of any further contact with a monocyte associated mitogen/antigen. The regulation of the response then is dependent on the presence or induction of cells having a suppressor effect on the B cell response. At least two suppressor cells have been recognized - suppressor monocytes and suppressor T cells. Recent work has indicated that the target for the latter is the helper T cell. Arguments pertaining to the action and evolution of suppressor T cells can equally well be used in describing the same features in suppressor monocytes. It is not known how suppressor cell activity arises. Monoclonal antibody studies indicate that these cells can be recognized in normal peripheral blood in the absence of activation by mitogen. While they can be enumerated, their functional activity has only recently been studied with these techniques. The way in which immunocompetent cells interact may

be envisioned in one of several ways:

1. A monocyte interacts with a helper T cell which in turn reacts with a B cell (to increase antibody production) and with a suppressor T cell (to increase suppressor activity which in turn limits further T cell help).

2. A monocyte interacts with a helper T cell which in turn activates a B cell (to increase antibody production) and a suppressor T cell (which inhibits antibody production by direct action on the B cell).

   This seems unlikely since recent evidence indicates that an action at this stage would not effectively block soluble helper factor-driven antibody production, and that T helper cells are necessary for the expression of T suppression.

3. A monocyte (or monocytes) interacts with both helper T cells and suppressor T cells. The helper T cells then act on B cells (to increase antibody production) while suppressor T cells subsequently act on these helper T cells to provide inhibition of further T help.

4. A monocyte interacts with a T cell which initially helps i.e. activates a B cell (increasing antibody production) and then transforms into a suppressor cell, which in turn acts on other helper cells to inhibit further response.

This postulate attempts to account for the observations that T lymphocytes may transform between Tγ and Tμ cells[289] and that $OKT4^+$ cells may generate suppressor activity independent of $OKT8^+$ cells[363].

The number of possible permutations increases substantially once subsets of helper and suppressor monocytes are also considered in the sequence. Any of these types of regulation mayube achieved through changes in the number of cells or in their functional activities. Enumeration experiments conducted in normals indicate that differences between individuals cannot be accounted for on the basis of variation in the number of cells, i.e. the number of Tγ cells did not correlate with the enhancement factor. Thus the response appears to be regulated by changes in the activites of cells and/or their interaction with other cells rather than by changes in numbers - a feature offering obvious biologic advantage in a system which requires to be flexible, sensitive and efficient. If Ia positivity denotes an active cell then a small but significant proportion of T cells are normally active, indicating that the immune system is always in a state of readiness rather than being turned "on" and "off" (as it is often loosely termed). This concept is reinforced by the observation that with in vitro activation in

culture systems, the number of Ia positive cells increases and soluble factors (some of which are Ia positive) are released into the supernatant.

While little can be said from these studies regarding monocyte function, it is evident that monocytes travel with B cells through the separation procedure, some being lost on the nylon wool column (unseparated and B cell preps have equivalent numbers of monocytes). It is evident that this cell is assuming progressively greater importance in the immune response both as a regulator and an effector. In addition to using current methodologies to dissect T subpopulations, it is necessary to also evaluate monocyte (and possibly B cell) subpopulations. Initial work suggests that feedback systems similar to those which control hormone synthesis and secretion, exist in the immune system. The method by which communication is mediated, may be cell-cell interaction, soluble factors or both. These soluble factors cross allogeneic boundaries and once released from helper T cells their effect cannot be blocked by suppressor T cells. Therefore, at least part of the action of suppressor T cells is to inhibit the release of soluble helper factors by helper T cells.

Monoclonal antibodies have allowed better definition of T cell subsets, than was previously available. It is evident that 80% of peripheral lymphocytes are T cells of which 50-60% are helper cells and 20-30% suppressor cells. Since regulation is

apparently independent of the number of cells of a particular functional subset, it is unclear why there is preponderance of helper T cells. This may relate to the efficiency of the immune response which is geared to a positive rather than a negative response, and where initiation is dependent on an antigen bearing monocyte contacting a helper T cell. The larger propotion of helper cells may favour the speed and probability with which this interaction occurs and thus offer biologic advantage.

It has been recognized that too much help may result in suppression. Thus after a critical point as more radiated (T_{3200}) cells are added, the response fails. While this may be due to nutrient exhaustion, it has been observed that cell crowding in several systems, results in the release of soluble factors having an inhibitory role. If the immune response were mediated by a vast increase in the number of immuno-competent cells, then the net result might be suppression rather than help. Thus modulation by a change in cell activity or communication rather than by recruitment of numbers may be important in maintaining the integrity of the response. It is not clear how these in vitro observations, relate to the in vivo situation of a chronic inflammatory infiltrate of lymphocytes and plasma cells. However, it possibly explains in part why inflammatory infiltrates and granulomas are finite in size.

The normal immune system can be visualized as a finely tuned network of communicating immuno-competent cells, having the ability to respond in a specific but controlldd fashion to a wide range of antigens. While homeostasis has been traditionally described in terms of a straight line, studies conducted on biochemical, phsiological and cellular functions in humans, animals and plants have indicated that in the steady state homeostasis follows a rhythmic fluctuation which is of a variable period but often circadian. The signal/noise ratio is often small and therefore it is unlikely that this type of variation could be recognized with current methodologies.

In contrast to these observations in normal subjects, subjects with rheumatoid arthritis differed in their responses. While PFC responses in cultures of unseparated lymphocytes, B cells alone, T cells alone and B + nonirradiated T cells in 1:1 ratio were normal, the enhancement seen when B cells were cocultured with T_{3200} cells in normals was absent in rheumatoids. This difference can be explained in several ways:

1. Decreased number of B lymphocytes.
2. Hyporesponsiveness of a normal number of B lymphocytes.
3. Decreased number of suppressor T cells.
4. Hyporesponsiveness of a normal number of suppressor T cells.
5. Decreased number of helper T cells.

6. Hyporesponsiveness of a normal number of helper T cells.
7. Defective communication between functionally normal cells.
8. Defective number or function of monocytes.

In respect of the number of B cells, it is known that rheumatoids have a normal number of peripheral blood B cells. B cell yields following separation procedures were similar in rheumatoids and normals suggesting that the difference could not be accounted for on the basis of a reduction in the number of B lymphocytes in peripheral blood or prepared cell suspensions in rheumatoids. Furthermore, since $5x10^4$ cells were cultured, any difference in the original numbers would have been corrected in culture preparations.

RAB cells did not appear to be hyporesponsive since in studies 1-6, PFC responses from cultures of B cells alone, B + non-irradiated T (1:1 ratio) and unseparated lymphocytes, were similar in rheumatoids and normals. Furthermore, when rheumatoid B cells received adequate helper signals either from allogeneic normal T_{3200} cells or (Chapter 9) excessive numbers of autologous RA T_{3200} cells (Chapter 11), PFC responses of a normal magnitude were observed, indicating the intrinsic functional normality of rheumatoid B lymphocytes.

Suppressor T cells were enumerated by two techniques, Tγ rosetting and OKT5 monoclonal antibody. The former method has its limitations as previously mentioned. However, these limitations are operative whether enumerating T cells from normal subjects or from rheumatoids. No difference was observed between rheumatoids and normals although ranges were described for both groups providing the initial evidence that no numerical differences existed between the numbers of suppressor T cells in the two groups. By a different and more sophisticated technique ranges were described and again there was no difference between rheumatoids and normals. However, a difference was observed when only those patients with active disease were considered. A decrease in the number of T suppressor cells in these patients resulted in an increase in the help (OKT4): suppression (OKT5) ratio favouring help. Further study is required to understand the significance of this observation. Since the numbers of Tγ cells did not correlate with radiation enhancement in rheumatoids or normals, it was clear that immunoregulation was independent of numbers.

The function of suppressor T cells was studied directly, but review of the data indicates that results could not be explained on the basis of an isolated dysfunction of this T cell subset. If the only problem was one of hyporesponsiveness of suppressor T cells then PFC responses of rheumatoid cultures containing B + non-irradiated

T cells would be higher than corresponding cultures from normals due to unopposed helper activity in the former. Then, when T cells were pre-irradiated at 3200 rads little increase would be seen above the already elevated PFC levels in rheumatoids, while significant enhancement to a comparable level would be noted in normals. This type of response was observed by my colleagues d.b. and e.c. in concurrent studies, which they were conducting using the same techniques on patients with systemic lupus erythematosus (SLE). This not only illustrates that differences exist between patients with rheumatoid arthritis and SLE but also that the techniques used here were able to detect these differences. This assay can only recognize suppression in the context of help. Therefore, if suppression is removed and help is normal the result is an increase in the PFC response. However, if help is also defective then when suppression is removed by radiation or Tγ depletion there is no augmentation of the response (an effect which may be interpreted as (a) pure restriction of B cell responsiveness, (b) pure restriction of helper T cell activity, (c) a combined defect of helper and suppressor T cell activity or (d) a failure of cell communication).

The number of helper T cells as determined by OKT4 monoclonal antibody was the same in rheumatoids and normals indicating an adequacy in the number of cells having helper activity.

Since PFC responses were enhanced when B cells were cocultured with non-irradiated T cells it is evident that rheumatoid T cells can provide some help, albeit restricted. Evidence from these studies suggests that rheumatoid T cell function is restricted and that this restriction can be overcome by an increase in numbers:

1. Functional destruction or physical removal of suppressor T cells is not followed by an increase in immunoglobulin synthesis in R.A.
2. RA T_{3200} cells are unable to enhance the PFC response of normal B cells to the extent observed with allogeneic or autologous normal RA T_{3200} cells.
3. Four times as many RA T_{3200} cells are required to maximize the PFC response in rheumatoids than in normals.

These observations are consistent with the concept that each cell is able to provide only a fraction of the helper activity of normal cells. While the problem may be a pure functional deficiency of helper T cell activity it is also conceivable that a combined functional deficiency of helper and suppressor T cell or defective communication between cells can explain the results. These possibilities cannot be completely dissected with the techniques used. Since other researchers, using different techniques, have described defects in suppressor

T cell activity, it seemed reasonable to conclude that both T help and T suppression are likely abnormal in rheumatoid arthritis. It cannot be known from available evidence whether these defects are independent of one another, or whether one is a regulatory response to the other e.g. hypofunction of suppressor T cells results in a compensatory reduction in T helper cell function in order to maintain homeostasis. In respect of helper T cells, since restricted helper activity may be overcome by increasing the number of T_{3200} cells in a culture, and since in normals this would produce a decline in the response, this evidence may favour an intrinsic abnormality of helper T cells rather than a communication problem between cells. However, more direct and conclusive evidence is not available to date.

Certain judgements regarding the numerical and functional normality of monocytes have been made in this thesis, some on the basis of indirect evidence. In order to comprehend the role of this cell in the PFC response, further work is required, an action which appears justified on the basis of recent evidence regarding the pivotal role that this cell apparently plays in many immune responses. In the last few days, Janossy et al[368], have reported their observations on rheumatoid synovium. Large, very strongly HLA DR positive macrophage-like interdigitating cells were noted to form close contact with $ORT4^{+}$ cells, while

the $OKT8^+$ population between these macrophage-$OKT4^+$ clusters were scanty (T4/T8 ratio = 9:1). This phenomenon was not observed in normal or osteoarthritic synovium or normal lymph node tissue. The authors suggested that HLA DR macrophage-like cells might process antigen and then attract reactive $OKT4^+$ cells to form clusters. The combination of monocytes, $OKT4^+$ cells and B cells would provide the necessary interaction for the production of immmnoglobulin and rheumatoid factor. The immunoregulatory defect produced by a relative deficiency of $OKT8^+$ cells in this area would allow perpetuation of immuno-globulin synthesis (N.B. OKT5 and OKT8 are both markers for cells thought to have suppressor activity).

Abnormalities noted in regulation of the immune response in rheumatoids may be attributed to one of several mechanisms:

1. A primary event having aetiologic or pathogenetic importance.
2. A phenomenon secondary to the disease.
3. A phenomenon secondary to treatment i.e. drug effect.

To have aetiologic importance the immune abnormalities noted must precede the presence of the clinical condition. This question cannot be answered by present studies, but may be approached in one of two ways, firstly with family studies and secondly with a cohort study. In the former one may search for similar abnormalities in genetically identical relatives who do not have the disease and infer

that an association exists. In the latter, individuals are assessed at time 0, their immune responses defined and they are then followed for the development of rheumatoid arthritis. Given the high cost and labour intensive nature of the work involved, and the known frequency of rheumatoid arthritis, the former approach is preferable.

The potential pathogenetic importance can be appreciated if the in vitro nature of the investigation is taken into account. Rheumatoid arthritis is characterized by an activated immune system which produces hypergammaglobulinaemia and autoantibodies. How then can this in vivo activation be reconciled with the restricted activity noted in vitro? Evidence suggests that the ability of a given cell to respond to antigen is not limitless, i.e. response to one antigen compromises the ability to respond to another antigen. It is often said that these cells are preoccupied or committed. It seems likely that these in vivo and in vitro observations can be encompassed within this explanation, i.e. the extent to which rheumatoid lymphocytes can respond in vitro is restricted by virtue of a prior response to another antigen (in vivo), although their PFC responsiveness is not absent. Since cells were washed several times during the procedure it was evident that serum factors or loosely adherent biologically active molecules were not the principle mediators of this restricted activity but rather, that it was an intrinsic defect in the cell or its mode of communication, inflicted by prior antigen exposure in the disease state.

As to the question of whether the abnormalities seen were primary or secondary, this could not be answered from these studies. It is an extremely difficult fact to establish. The absence of regulatory abnormalities in genetically identical relatives without rheumatoid arthritis (in a family study), cannot be taken as satisfactory evidence of its secondary nature. Following patients with disease in order to observe whether changes in the degree of abnormality precede or follow changes in the activity of disease is problematic, in view of the inherent variability in test methods, and in measurement of the time of such a change in disease activity. No correlation was observed in these studies however, between activity and defective immuno-regulation, with the exception of alteration of OKT4/OKT5 ratios favouring help in patients with active disease. However, it was not known whether these changes occurred before or after the disease "flare". Since only one measurement in time was made in each individual the significance of this finding was unclear. A final and equally problematic method is to study patients before and after the onset of disease. Since we are unable to define when rheumatoid arthritis starts but only know when it is clinically evident, it is unlikely that this approach would be successful.

The question as to whether a difference between two groups is due to the presence or absence of disease or to some other factor, is a constant challenge to the interpretation of between-group differences. A common problem is that of knowing whether drug

therapy has produced differences which would not otherwise have been discovered. In view of the nature of rheumatoid arthritis and the necessity to treat the pain and disability which accompany it, it is exceedingly difficult to find patients with active disease, who are not on treatment with anti-inflammatory drugs. While the washout time for some drugs is short (nonsteroidal anti-inflammatory drugs), for others it is prolonged (gold, D-Penicillamine and Chloroquine). It is not known how long a washout period is necessary before remaining abnormalities in lymphocyte function can be ascribed to non-drug factors. Occasionally one encounters patients who have not been treated or are on analgesics not known to affect lymphocyte function. However, these patients are in the minority. An alternative approach is to study patients on different doses of different drugs, and see if there is any association between any one drug or drug type. The studies previously described, have noted no such correlation, suggesting that the observed phenomena likely cannot be ascribed to drug therapy. Furthermore in the final experiment entirely normal enhancement was observed in a normal subject taking Motrin 600 mgs per day. However, to directly address this question, the study of drug-free rheumatoids is essential.

In respect of the original research question, a defect in immunoregulation has been identified in vitro in patients with rheumatoid arthritis i.e.-

1. Immunoglobulin synthesis failed to enhance normally, when suppressor T cells were functionally destroyed by optimal dose radiation.

2. Immunoglobulin synthesis failed to enhance normally, when suppressor T cells were physically removed by Tγ rosetting.

The nature of the defect was not completely elucidated. However, experimental results suggested that it was due to dysfunction of helper and possibly suppressor T cells, rather than dysfunction of B cells or monocytes, or a numerical abnormality of any cell type since:+

1. Immunoglobulin synthesis was enhanced by T cells but not further enhanced by irradiation or removal of suppressor T cells.
2. R.A. T_{3200} cells failed to normally enhance immunoglobulin synthesis from normal B cells.
3. Four times as many R.A. T_{3200} as normal T_{3200} cells were required to produce normal radiation enhancement.
4. Recent work from other laboratories has defined abnormalities in suppressor T cells.
5. While functional normality of monocytes has been inferred from a single experiment, this mononuclear cell subset requires additional study.
6. The numbers of Tγ, $OKT4^+$ and $OKT5^+$ cells were normal in rheumatoid cell suspensions, with the exception of an increase in the OKT4/OKT5 ratio in active patients.

It is evident from the foregoing discussion, that at this point in the history of immunology, remarkably progress is being made in areas central to comprehension of the maintenance of the immune milieu in healthy subjects, the mechanism by which a controlled and specific response is mounted to foreign antigens, and in the appreciation of those aspects of immunopathology by which regulatory failure leads to chronic disease states. While much use has been made of new technology in the quest for knowledge, the power of the human intellect should not be underestimated. This is well illustrated by an observation made by Warner[79]. Some seventy years before the advent of the scanning electron microscope, Paul Ehrlich illustrated a paper on his side chain theory, with a remarkably accurate drawing of the surface of the B lymphocyte. (Figure 41).

Perhaps the greatest single advance of recent years has been in hybridoma technology. Monoclonal antibodies allow researchers to work with cell preparations of much higher purity than was previously possible. When coupled with the immunofluorescent cell sorter these antibodies provide the tools with which to dissect a complex immune network. The results of studies reported in this thesis provide several avenues for further research.

1. The extent to which cells maintain their functional integrity (i.e. Tγ and Tμ cells)urgently requires closer examination, since it affects study design and data interpretation and may represent a key element in the regulation of the immune response.

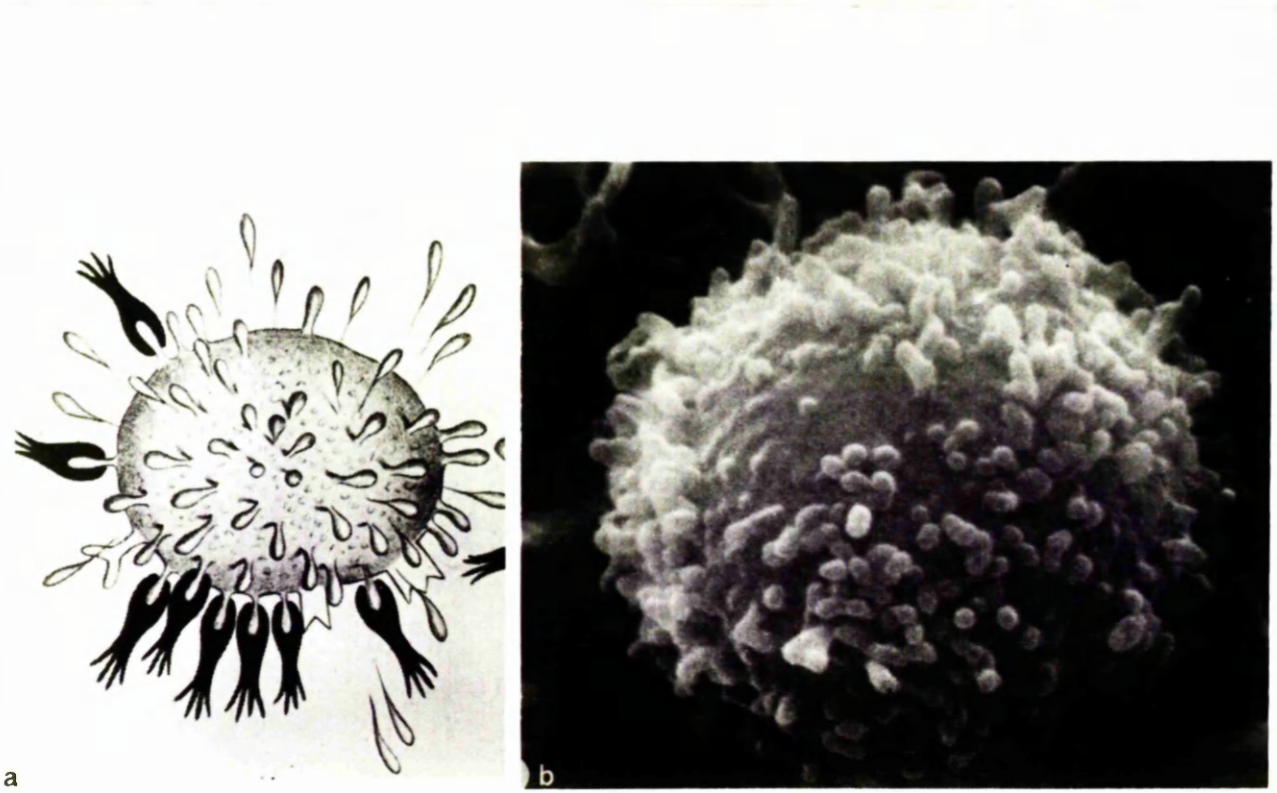


FIGURE 41 - COMPARISON OF PAUL EHRLICH'S IMPRESSION OF THE CELL SURFACE OF A B LYMPHOCYTE (c 1900) WITH A SCANNING ELECTRON MICROGRAPH (POLLIACK 1973). COURTESY OF N.L. WARNER[79]

2. The significance of Ia positivity in lymphocytes and monocytes requires further evaluation, since cells bearing Ia antigens continue to be recognized in the inductive phase of some immune responses.
3. There is a need to conduct further studies on young early drug-free rheumatoids using the methods detailed in this thesis in order to completely rule out any confounding effect of therapy.
4. It is necessary to conduct further functional studies with purified T cell subsets. Thus, rather than using irradiated T_{3200} cells, responses to increasing numbers of $OKT4^+$ cells should be studied. Furthermore crossover experiments using RA B cells and normal $OKT4^+$ cells would provide additional information regarding the normality of the rheumatoid B cell.
5. Radiation dose response curves should be constructed for OKT4 and OKT5 populations in order to describe the spectrum of radiosensitivity. Enumeration of Tγ cells in $OKT5^+$ cell suspensions would allow further examination of the relationship between these two "markers" for suppressor cells.
6. Functional subsets of monocytes require better definition. Present evidence indicates that monocytes may play helper as well as suppressor roles in the immune response.

7. If 1-6 can be achieved, then it may be possible to perform mixing experiments with pure functional subsets of several classes of mononuclear cells and define immunoregulatory disturbances in diseased individuals. This assumes that (a) subsets maintain their functional integrity and (b) in vitro observations reflect in vivo events.

Continuing interest in this area of research in the next several years can be anticipated to yield essential information, provide a most satisfying intellectual challenge to the researcher, and eventually allow the clinician a greater insight into the enigma called rheumatoid arthritis.

APPENDICES

APPENDIX 1. PATIENT PROFILES.

Rheumatoid Arthritis:

AT 50 year old female from Wallaceburg, Ontario. Disease duration 15 years. ESR 49. Rheumatoid factor 1/160. Functional class 2. Drug therapy at time of study: Prednisone 10 mg daily. ARA diagnostic criteria 1, 2, 3, 4, 5, 8. No x-rays were available on this patient.

BF 64 year old male from Blenheim, Ontario. Disease duration 16 years. ESR 24, rheumatoid factor 1/320. Presence of radiographic erosion. Drug therapy at time of study: Entrophen 975 mg p.o. q.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8, 9.

BL 59 year old female from Wallaceburg, Ontario. Disease duration 17 years. ESR 66, rheumatoid factor 1/320. Presence of radiographic erosion. Drug therapy at time of study: Motrin 2.4 gms per day, Prednisone 5 mg p.o. once daily. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

CF 70 year old male from Windsor, Ontario. Disease duration 2 years. ESR 44. Rheumatoid factor 1/160. No erosions on x-ray. Functional class 2. Drug therapy at time of study: Entrophen 975 mg p.o. q.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

CK 70 year old male from Port Dover, Ontario. Disease duration 15 years. Rheumatoid factor 1/1280. ESR 54. Severe erosions present on x-ray. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8, 9, 10. Drug therapy at time of study: Motrin 400 mg p.o. q.i.d. Tylenol #2 p.r.n. Functional class 3.

CM 62 year old female from Mount Forest, Ontario. Disease duration 27 years. ESR 34, rheumatoid factor 1/160. Erosions present. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 7, 8, 9. Functional class 3. Drug therapy at time of study: Penicillamine 250 mg p.o. once daily, Prednisone 7.5 mg daily, Entrophen 4.5 gms per day. This patient has also been noted to have vasculitis in the past.

CS 71 year old female from London, Ontario. Disease duration 2-1/2 years. ESR 18. Rheumatoid factor 1/320. Probable erosions on x-ray. Functional class 2/3. Drug therapy at time of study: Motrin 600 mg q.i.d., Prednisone 5 mg q.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

DB 56 year old female from Camlachie, Ontario. Disease duration 10 months. ESR 46. Rheumatoid factor 1/320. No erosions. ARA diagnostic criteria 1, 2, 3, 4, 5, 8. Functional class 3. Drug therapy at time of study: Orudis 50 mg p.o. q.i.d.

DK 47 year old female from London, Ontario. Disease duration 8 years. ESR 13. Rheumatoid factor 1/80. No erosions on x-ray. ARA diagnostic criteria 1, 2, 3, 4, 5, 8. Drug therapy at time of study: Myochrisine. Functional class 2.

DM 56 year old female from Beachville, Ontario. Disease duration 8 years. ESR 42. Rheumatoid factor 1/1,280. No erosions. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Functional class 2. Drug therapy at time of study: Entrophen 2.4 gms per day, Indocid 75 mg. at h.s.

DM_1 31 year old female from London, Ontario. Disease duration 8 years. ESR 42. Rheumatoid factor 1/1,280. No erosions in x-ray. Functional class 2. Drug therapy at time of study: Penicillamine 1 gm per day. Naprosyn 1 gm per day, Prednisone 5 mg per day. ARA diagnostic criteria 1, 2, 3, 4, 5, 8.

DN 33 year old female from Stratford, Ontario. Disease duration 18 months. ESR 40. Rheumatoid factor 1/2,560. Erosions on x-ray. Functional class 2. Drug therapy at time of study: Naprosyn 750 mg daily. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 7, 8.

EB 39 year old female from Wallaceburg, Ontario. Disease duration 11 years. ESR 14. Rheumatoid factor less than 1/20. No erosions. ARA diagnostic criteria 1, 2, 3, 4, 5. Drug therapy at time of study: Tylenol #1, Maxeran 10 mg t.i.d. Tagamet 300 mg q.i.d. Tofranil 75 mg at h.s. Functional class 1.

EL 85 year old female from Forest, Ontario. Disease duration 45 years. Rheumatoid factor 1/640. ESR 32. Erosions are present on x-ray. Functional class 4. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Drug therapy at time of study: Naprosyn 375 mg p.o. b.i.d., Digoxin, Reserpine and Secobarbital.

EM 68 year old female from Woodstock, Ontario. Disease duration 6 weeks. ESR 56. Rheumatoid factor 1/320. No x-rays available. Functional class 2. Drug therapy at time of study: Orudis 50 mg t.i.d., Entrophen p.r.n. ARA diagnostic criteria 1, 2, 3, 4, 5, 8.

FM 57 year old female from Windsor, Ontario. Disease duration 14 years. Rheumatoid factor 1/40. ESR 24. Erosions present on x-ray. Functional class 3. ARA diagnostic criteria 1, 2, 3, 4, 5, 7. Drug therapy at time of study: Entrophen and Chloroquine.

GW 53 year old female from Sarnia, Ontario. Disease duration 21 years. ESR 32. Rheumatoid factor 1/160. Erosions present on x-ray. Functional class 2. Drug therapy at time of study: Dexamethysone 0.75 mg b.i.d., Entrophen 650 mg t.i.d., and Nalfon. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

HB 54 year old female from Windsor, Ontario. Disease duration 23 years. ESR 39. Rheumatoid factor 1/10,240. Erosions present. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Functional class 1. Drug therapy at time of study: Entrophen 3 gms per day, Prednisone 5 mg daily, Penicillamine 500 mg per day.

HL 66 year old female from Dresden, Ontario. Disease duration 20 years. ESR 56. Rheumatoid factor 1/320. Erosions present on x-ray. Functional class 3. Drug therapy at time of study: Penicillamine 375 mg per day, Indocid 100 mg q.h.s. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 7, 8.

HM 56 year old female from Blenheim, Ontario. Disease duration 10 years. ESR 50. Rheumatoid factor 1/2,560. Radiographic erosions present. Drug therapy at time of study: Entrophen 975 mg p.o. q.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 7, 8, 9. Functional class 2.

JC 46 year old female from London, Ontario. Disease duration 16 years. ESR 40, rheumatoid factor less than 1/20. Erosions present on x-ray. Functional class 2. Drug therapy at time of study: 4.8 gms of Aspirin per day. ARA diagnostic criteria 1, 2, 3, 4, 5, 7.

JF 79 year old female from Petrolia, Ontario. Disease duration 6 months. ESR 56. Rheumatoid factor less than 1/20. Evidence of radiographic erosion. Drug therapy at time of study: Motrin 400 mg p.o. q.i.d., Quinidine 100 mg p.o. q.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 9.

JG 57 year old female from Windsor, Ontario. Disease duration 8 years. ESR 54. Rheumatoid factor 1/10,240. Erosions present on x-ray. Functional class 4. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Drug therapy at time of study: Naprosyn 375 mg p.o. b.i.d., Indomethacin 75 mg p.o. q.h.s., Myochrysine 50 mg i.m. once weekly.

JL 33 year old female from Courtwright, Ontario. Disease duration 5 years. ESR 48. Rheumatoid factor 1/320. No erosions on x-ray. Functional class 2. Drug therapy at time of study: Prednisone 2.5 mg., Penicillamine 500 mg per day. ARA diagnostic criteria 1, 2, 3, 4, 5, 8, 9.

LH 31 year old female from Stratford, Ontario. Disease duration 4 years. ESR 13, rheumatoid factor 1/160. Erosions present on x-ray. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Functional class 2. Drug therapy at time of study: Entrophen 4.2 gms daily, Indocid 50 mg h.s.

LL 60 year old female from London, Ontario. Disease duration 6 years. ESR 51, rheumatoid factor 1/5,120. Radiologic erosions present. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 7, 8. Functional class 2. Drug therapy at time of study: 6 22's per day.

MB 59 year old female from Delhi, Ontario. Disease duration 25 years. ESR 66. Rheumatoid factor 1/1280. Erosions present on x-ray. Functional class 3/4. Drug therapy at time of study: Tolectin 1200 mg daily. This patient also had chronic renal failure and pernicious anemia. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

MG 60 year old female from Cotham, Ontario. Disease duration 20 years. ESR 54. Rheumatoid factor 1/640. Erosions present on x-ray. Functional class 3. Drug therapy at time of study: Tolectin 1200 mg daily, Penicillamine 750 mg daily, Prednisone 10 mg per day. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

MK 74 year old female from Wallaceburg, Ontario. Disease duration 2 years. ESR 7, rheumatoid factor 1/1,280. No erosions. ARA diagnostic criteria 1, 2, 3, 4, 5, 6, 8. Functional class 2. Drug therapy at time of study: Chloroquine 250 mg per day.

MK_1 64 year old female from London, Ontario. Disease duration 34 years. Rheumatoid factor 1/1280. ESR 24. Erosions present on x-ray. Functional class 2/3. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8, 9. Drug therapy at time of study: Penicillamine, Indomethacin

ML 33 year old female from Windsor, Ontario. Disease duration 13 years. ESR 22. Rheumatoid factor 1/180. No erosions. Functional class 2. Drug therapy at time of study: Voltoran 50 mg p.o. t.i.d. Gold 50 mg weekly. ARA diagnostic criteria 1, 2, 3, 4, 5, 8. Coexistant problem keratomalacia.

MS 62 year old female from Tillsonburg, Ontario. Disease duration 20 years. ESR 49. Rheumatoid factor 1/1,280. Erosions present on x-ray. Functional class 2. Drug therapy at time of study: Penicillamine 250 mg: Indocid 100 mg at h.s., Naprosyn 375 mg b.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

MS_1 60 year old female from St. Thomas, Ontario. Disease duration 36 years. ESR 30. Rheumatoid factor 1/320. Erosions present on x-ray. Functional class 3. Drug therapy at time of study: Prednisone 5 mg per day, Naprosyn 250 mg p.o. b.i.d. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

NI 57 year old female from Woodstock, Ontario. Disease duration 3 years. ESR 50. Rheumatoid factor 1/1280. Erosions present on x-ray. Functional class 1. Drug therapy at time of study: Entrophen 975 mg p.o. q.i.d.; ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

PL 55 year old female from Atwood, Ontario. Disease duration 21 years. Rheumatoid factor 1/20,480. ESR 52. Erosions present on x-ray. Functional class 3/4. Drug therapy at time of study: Indomethacin and Clinoril. This patient also had Sjogren's syndrome with increased C1Q binding and strongly positive ANA. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8, 9.

RM 49 year old female from Stratford, Ontario. Disease duration 13 years. ESR 37. Rheumatoid factor less than 1/20. No x-rays available. Functional class 2/3. Drug therapy at time of study: Entrophen 6 gms per day. ARA diagnostic criteria 1, 2, 3, 4, 5.

RR 63 year old male from Brantford, Ontario. Disease duration 20 years. ESR 50. Rheumatoid factor 1/320. Erosions present on x-ray. Functional class 2. Drug therapy at time of study: Telectin 1200 mg daily. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Patient also had mild chronic renal failure and Sjogren's syndrome.

SN 61 year old male from Aylmer, Ontario. Disease duration 4 months. ESR 28. Rheumatoid factor less than 1/20. Erosions present on x-ray. Functional class 1. Drug therapy at time of study: Entrophen 3.6 gms per day. ARA diagnostic criteria 1, 2, 3, 4, 5, 7.

SS 86 year old female from Tavistock, Ontario. Disease duration 5 months. ESR 50. Rheumatoid factor 1/5,120. No erosions. ARA diagnostic criteria 1, 2, 3, 4, 5, 8. Functional class 2. Drug therapy at time of study: Entrophen 975 mg p.o. q.i.d.

SW 59 year old male from Windsor, Ontario. Disease duration 4 months. ESR 48. Rheumatoid factor 1/1,280. Erosions present on x-ray. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8, 9. Functional class 1. Drug therapy at time of study: Entrophen 975 mg p.o. q.i.d.

SW_1 57 year old female from London, Ontario. Disease duration 11 years. ESR 42. Rheumatoid factor 1/640. Questionable erosions present on x-ray. Drug therapy at time of study: Entrophen 3.2 gms per day. ARA diagnostic criteria 1, 2, 3, 4, 5, 8.

TM 49 year old female from London, Ontario. Disease duration 5 years. ESR 46. Rheumatoid factor 1/5,120. Erosions present on x-ray. Functional class 3/4. Drug therapy at time of study: Prednisone 5 mg once daily, Entrophen 975 mg q.i.d., Indocid 100 mg at h.s., Imuran 75 mg once daily. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8.

US 46 year old female from London, Ontario. Disease duration 3 months. ESR 41. Rheumatoid factor 1/160. X-rays not done. Functional class 2. Drug therapy at time of study: Tolectin 400 mg t.i.d., Indocid 50 mg at h.s. ARA diagnostic criteria 1, 2, 3, 4, 5, 8.

VC 57 year old female from La Salette, Ontario. Disease duration 24 years. ESR 32. Rheumatoid factor 1/640. Erosions present on x-ray. Functional class 3. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8. Drug therapy at time of study: Motrin 600 mg p.o. q.i.d.

WS 61 year old male from Tavistock, Ontario. Disease duration 2 weeks. ESR 33. Rheumatoid factor 1/640. No erosions on x-ray. Drug therapy at time of study: Motrin 600 mg p.o. q.i.d. Prednisone 7.5 mg daily. Functional class 2. ARA diagnostic criteria 1, 2, 3, 4, 5, 7, 8, 9.

APPENDIX 2. PROFILES OF NORMAL SUBJECTS. *

ac 27 year old male physician

bt 42 year old female secretary

bw 57 year old female secretary

ce 32 year old male research assistant

ce_1 43 year old female secretary

ch 28 year old female nurse

cm 24 year old female medical student

cm_1 24 year old female lab technician

cp 29 year old male physician

cr 21 year old female medical student

db 42 year old male physician

db_1 28 year old male physician

db_2 21 year old male medical student

ds 29 year old female research assistant

ds_1 25 year old male physician

ec 30 year old female research assistant

ej 52 year old female nurse

hk 60 year old female secretary

jd 31 year old male lab technician

jh 30 year old male lab technician

jo 34 year old male lab technician

jw 59 year old female secretary

ke 31 year old female research assistant

lg	28 year old physician
ma	23 year old male research student
me	42 year old female secretary
mf	53 year old female secretary
mm	23 year old male research student
nb	30 year old male physician
nl	29 year old female physician
ph	61 year old female secretary
ry	27 year old male physician
wc	38 year old male research worker
ww	31 year old male medical student

* All subjects from London, Ontario.

APPENDIX 3. ORIGINAL DATA TABLES

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
o.c.	1	B	+	(10^5)	-	-	-	0	1	0
	2	B	-	(10^5)	-	-	-	0	2	0
	3	B & T	+	($5x10^4$)	($5x10^4$)	-	-	0	3	10
	4	B & T	-	($5x10^4$)	($5x10^4$)	-	-	0	4	5
	5	T	+	-	(10^5)	-	-	0	5	0
	6	T	-	-	(10^5)	-	-	0	6	0
	7	B	+	10^5	-	-	-	3	7	2480
	8	B	+	10^5	-	-	-	3	8	1980
	9	B	-	10^5	-	-	-	3	9	320
	10	B	-	10^5	-	-	-	3	10	200
	11	T	+	-	10^5	-	-	3	11	40
	12	T	+	-	10^5	-	-	3	12	20
	13	T	-	-	10^5	-	-	3	13	40

Subject	Culture	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
e.c.	14	T	-	-	10^5	-	-	3	14	20
	15	B & T	+	$5x10^4$	$5x10^4$	-	-	3	15	1830
	16	B & T	+	$5x10^4$	$5x10^4$	-	-	3	16	1330
	17	B & T	-	$5x10^4$	$5x10^4$	-	-	3	17	330
	18	B & T	-	$5x10^4$	$5x10^4$	-	-	3	18	340
	19	Unsep	+	-	-	10^5	-	3	19	2520
	20	Unsep	+	-	-	10^5	-	3	20	2680
	21	Unsep	+	-	-	10^5	-	3	21	2580
	22	Unsep	-	-	-	10^5	-	3	22	500
	23	Unsep	-	-	-	10^5	-	3	23	580
	24	B	+	10^5	-	-	-	7	24	2720
	25	B	+	10^5	-	-	-	7	25	2240
	26	B	-	10^5	-	-	-	7	26	620
	27	B	-	10^5	-	-	-	7	27	520
	28	T	+	-	10^5	-	-	7	28	180

Subject	Culture	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
e.c.	29	T	+	-	10^5	-	-	7	29	100
	30	T	+	-	10^5	-	-	7	30	40
	31	T	-	-	10^5	-	-	7	31	80
	32	T	-	-	10^5	-	-	7	32	40
	33	T	-	-	10^5	-	-	7	33	0
	34	T	-	-	10^5	-	-	7	34	20
	35	B & T	+	$5x10^4$	$5x10^4$	-	-	7	35	3140
	36	B & T	+	$5x10^4$	$5x10^4$	-	-	7	36	3240
	37	B & T	+	$5x10^4$	$5x10^4$	-	-	7	37	4020
	38	B & T	-	$5x10^4$	$5x10^4$	-	-	7	38	800
	39	B & T	-	$5x10^4$	$5x10^4$	-	-	7	39	740
	40	Unsep	+	-	-	10^5	-	7	40	3600
	41	Unsep	+	-	-	10^5	-	7	41	2000
	42	Unsep	-	-	-	10^5	-	7	42	400

Subject	Culture	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
e.c.	43	Unsep	-	-	-	10^5	-	7	43	320
	44	B	+	10^5	-	-	-	10	44	500
	45	B	+	10^5	-	-	-	10	45	450
	46	B	-	10^5	-	-	-	10	46	400
	47	B	-	10^5	-	-	-	10	47	250
	48	T	+	-	10^5	-	-	10	48	20
	49	T	+	-	10^5	-	-	10	49	80
	50	T	+	-	10^5	-	-	10	50	40
	51	T	-	-	10^5	-	-	10	51	20
	52	T	-	-	10^5	-	-	10	52	40
	53	B & T	+	$5x10^4$	$5x10^4$	-	-	10	53	1800
	54	B & T	+	$5x10^4$	$5x10^4$	-	-	10	54	1580
	55	B & T	-	$5x10^4$	$5x10^4$	-	-	10	55	320
	56	B & T	-	$5x10^4$	$5x10^4$	-	-	10	56	380
	57	Unsep	+	-	-	10^5	-	10	57	220

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
<u>e.c.</u>	58	Unsep	+	-	-	10^5	-	10	58	300
	59	Unsep	-	-	-	10^5	-	10	59	80
	60	Unsep	-	-	-	10^5	-	10	60	140
<u>c.e.</u>	61	B	+	(10^5)	-	-	-	0	61	200
	62	B	+	(10^5)	-	-	-	0	62	280
	63	B	-	(10^5)	-	-	-	0	63	220
	64	B	-	(10^5)	-	-	-	0	64	200
	65	T	+	-	(10^5)	-	-	0	65	60
	66	T	+	-	(10^5)	-	-	0	66	20
	67	T	-	-	(10^5)	-	-	0	67	40
	68	T	-	-	(10^5)	-	-	0	68	20
	69	B & T	+	($5x10^4$)	($5x10^4$)	-	-	0	69	380
	70	B & T	+	($5x10^4$)	($5x10^4$)	-	-	0	70	400

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.	71	B & T	-	($5x10^4$)	($5x10^4$)	-	-	0	71	360
	72	B & T	-	($5x10^4$)	($5x10^4$)	-	-	0	72	360
	73	Unsep	+	-	-	(10^5)	-	0	73	400
	74	Unsep	+	-	-	(10^5)	-	0	74	420
	75	Unsep	-	-	-	(10^5)	-	0	75	360
	76	Unsep	-	-	-	(10^5)	-	0	76	440
	77	B	+	10^5	-	-	-	3	77	1250
	78	B	+	10^5	-	-	-	3	78	750
	79	B	-	10^5	-	-	-	3	79	250
	80	B	-	10^5	-	-	-	3	80	350
	81	T	+	-	10^5	-	-	3	81	0
	82	T	+	-	10^5	-	-	3	82	0
	83	T	+	-	10^5	-	-	3	83	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.	84	T	-	-	10^5	-	-	3	84	0
	85	T	-	-	10^5	-	-	3	85	0
	86	T	-	-	10^5	-	-	3	86	50
	87	B & T	+	$5x10^4$	$5x10^4$	-	-	3	87	2000
	88	B & T	+	$5x10^4$	$5x10^4$	-	-	3	88	2275
	89	B & T	+	$5x10^4$	$5x10^4$	-	-	3	89	550
	90	B & T	-	$5x10^4$	$5x10^4$	-	-	3	90	925
	91	Unsep	+	-	-	10^5	-	3	91	550
	92	Unsep	+	-	-	10^5	-	3	92	400
	93	Unsep	-	-	-	10^5	-	3	93	400
	94	Unsep	-	-	-	10^5	-	3	94	600
	95	B	+	10^5	-	-	-	7	95	3100
	96	B	+	10^5	-	-	-	7	96	3750

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Reds.	Culture duration	Plaque No.	PFC/10^6 PBL
C.C.	97	B	-	10^5	-	-	-	7	97	2450
	98	B	-	10^5	-	-	-	7	98	1600
	99	T	+	-	10^5	-	-	7	99	650
	100	T	+	-	10^5	-	-	7	100	550
	101	T	-	-	10^5	-	-	7	101	400
	102	T	-	-	10^5	-	-	7	102	200
	103	B & T	+	$5x10^4$	$5x10^4$	-	-	7	103	4125
	104	B & T	+	$5x10^4$	$5x10^4$	-	-	7	104	4800
	105	B & T	-	$5x10^4$	$5x10^4$	-	-	7	105	750
	106	B & T	-	$5x10^4$	$5x10^4$	-	-	7	106	925
	107	Unsep	+	-	-	10^5	-	7	107	2950
	108	Unsep	+	-	-	10^5	-	7	108	3350
	109	Unsep	-	-	-	10^5	-	7	109	1150

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Reds.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.	110	Unsep	-	-	-	10^5	-	7	110	1100
	111	B	+	10^5	-	-	-	10	111	400
	112	B	+	10^5	-	-	-	10	112	500
	113	B	-	10^5	-	-	-	10	113	150
	114	B	-	10^5	-	-	-	10	114	50
	115	T	+	-	10^5	-	-	10	115	0
	116	T	+	-	10^5	-	-	10	116	0
	117	T	-	-	10^5	-	-	10	117	0
	118	T	-	-	10^5	-	-	10	118	50
	119	B & T	+	$5x10^4$	$5x10^4$	-	-	10	119	400
	120	B & T	+	$5x10^4$	$5x10^4$	-	-	10	120	300
	121	B & T	-	$5x10^4$	$5x10^4$	-	-	10	121	200
	122	B & T	-	$5x10^4$	$5x10^4$	-	-	10	122	300

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
e.e.	123	Unsep	+	-	-	10^5	-	10	123	200
	124	Unsep	+	-	-	10^5	-	10	124	400
	125	Unsep	-	-	-	10^5	-	10	125	200
	126	Unsep	+	-	-	10^5	-	10	126	50
e.c.	127	B	+(0.5%)	$5x10^4$	-	-	-	7	127	1150
	128	B	+(0.5%)	$5x10^4$	-	-	-	7	128	950
	129	B	+(0.5%)	$5x10^4$	-	-	-	7	129	700
	130	T	+(0.5%)	-	$5x10^4$	-	-	7	130	100
	131	T	+(0.5%)	-	$5x10^4$	-	-	7	131	0
	132	B & T	+(0.5%)	$5x10^4$	$5x10^4$	-	-	7	132	1090
	133	B & T	+(0.5%)	$5x10^4$	$5x10^4$	-	-	7	133	1330
S.W.	134	B	+(0.5%)	$5x10^4$	-	-	-	7	134	0
	135	B	+(0.5%)	$5x10^4$	-	-	-	7	135	179

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
S.W.	136	B	+(0.1%)	$5x10^4$	-	-	-	7	136	159
	137	B	+(0.1%)	$5x10^4$	-	-	-	7	137	159
	138	B	+(1%)	$5x10^4$	-	-	-	7	138	317
	139	B	+(1%)	$5x10^4$	-	-	-	7	139	159
	140	B	-	$5x10^4$	-	-	-	7	140	0
	141	B	-	$5x10^4$	-	-	-	7	141	286
	142	T	+0.01%	-	$5x10^4$	-	-	7	142	0
	143	T	+0.01%	-	$5x10^4$	-	-	7	143	0
	144	T	+0.1%	-	$5x10^4$	-	-	7	144	0
	145	T	+0.1%	-	$5x10^4$	-	-	7	145	0
	146	T	+ 1%	-	$5x10^4$	-	-	7	146	0
	147	T	+ 1%	-	$5x10^4$	-	-	7	147	0
	148	T	-	-	$5x10^4$	-	-	7	148	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
S.W.	149	T	-	-	$5x10^4$	-	-	7	149	0
	150	B & T	+0.01%	$5x10^4$	$5x10^4$	-	-	7	150	1430
	151	B & T	+0.01%	$5x10^4$	$5x10^4$	-	-	7	151	715
	152	B & T	+0.1%	$5x10^4$	$5x10^4$	-	-	7	152	2171
	153	B & T	+0.1%	$5x10^4$	$5x10^4$	-	-	7	153	2737
	154	B & T	+1%	$5x10^4$	$5x10^4$	-	-	7	154	3890
	155	B & T	+1%	$5x10^4$	$5x10^4$	-	-	7	155	4290
	156	B & T	-	$5x10^4$	$5x10^4$	-	-	7	156	1001
	157	B & T	-	$5x10^4$	$5x10^4$	-	-	7	157	143
M.F.	158	B & T	-	$5x10^4$	$5x10^4$	-	-	7	158	1275
	159	B & T	+0.1%	$5x10^4$	$5x10^4$	-	-	7	159	2475
	160	B & T	+0.5%	$5x10^4$	$5x10^4$	-	-	7	160	4275
	161	B & T	+1%	$5x10^4$	$5x10^4$	-	-	7	161	4937

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
m.f.	162	B & T	+1.5%	$5x10^4$	$5x10^4$	-	-	?	162	5050
	163	B & T	+ 2%	$5x10^4$	$5x10^4$	-	-	?	163	5025
	164	B & T	+ 3%	$5x10^4$	$5x10^4$	-	-	?	164	3562
p.h.	165	B & T	-	$5x10^4$	$5x10^4$	-	-	?	165	750
	166	B & T	+0.1%	$5x10^4$	$5x10^4$	-	-	?	166	2200
	167	B & T	+0.5%	$5x10^4$	$5x10^4$	-	-	?	167	4412
	168	B & T	+ 1%	$5x10^4$	$5x10^4$	-	-	?	168	5425
	169	B & T	+1.5%	$5x10^4$	$5x10^4$	-	-	?	169	4700
	170	B & T	+ 2%	$5x10^4$	$5x10^4$	-	-	?	170	4225
	171	B & T	+ 3%	$5x10^4$	$5x10^4$	-	-	?	171	2500
c.e.	172	B	+(RM)	$5x10^4$	-	-	-	?	172a	600
	[illegible]	B	+(RM)	$5x10^4$	-	-	-	?	172b	350
	173	B	-	$5x10^4$	-	-	-	?	173a	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.		B	+(RM)	$5x10^4$	-	-	-	7	173b	100
	174	T	+(RM)	-	$5x10^4$	-	-	7	174a	125
		T	+(RM)	-	$5x10^4$	-	-	7	174b	0
	175	T	-	-	$5x10^4$	-	-	7	175a	0
		T	-	-	$5x10^4$	-	-	7	175b	0
	176	B & T	+(RM)	$5x10^4$	$5x10^4$	-	-	7	176a	3780
		B & T	+(RM)	$5x10^4$	$5x10^4$	-	-	7	176b	3500
	177	B & T	-	$5x10^4$	$5x10^4$	-	-	7	177a	415
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	177b	498
	178	Unsep	+(RM)	-	-	10^5	-	7	178a	4905
		Unsep	+(RM)	-	-	10^5	-	7	178b	4815
	179	Unsep	-	-	-	10^5	-	7	179a	53
		Unsep	-	-	-	10^5	-	7	179b	53

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.	180	B	+(GIB)	$5x10^4$	-	-	-	7	180a	781
		B	+(GIB)	$5x10^4$	-	-	-	7	180b	1136
	181	B	-	$5x10^4$	-	-	-	7	181a	213
		B	-	$5x10^4$	-	-	-	7	181b	284
	182	T	+(GIB)	-	$5x10^4$	-	-	7	182a	62
		T	+(GIB)	-	$5x10^4$	-	-	7	182b	62
	183	T	-	-	$5x10^4$	-	-	7	183a	82
		T	-	-	$5x10^4$	-	-	7	183b	82
	184	B & T	+(GIB)	$5x10^4$	$5x10^4$	-	-	7	184a	3500
		B & T	+(GIB)	$5x10^4$	$5x10^4$	-	-	7	184b	5250
	185	B & T	-	$5x10^4$	$5x10^4$	-	-	7	185a	284
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	185b	568
	186	Unsep	+(GIB)	-	-	10^5	-	7	186a	8549

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.		Unsep	+(GIB)	-	-	10^5	-	7	186b	5229
	187	Unsep	-	-	-	10^5	-	7	187a	92
		Unsep	-	-	-	10^5	-	7	187b	92
	188	B	+(FLOW)	$5x10^4$	-	-	-	7	188a	1125
		B	+(FLOW)	$5x10^4$	-	-	-	7	188b	625
	189	B	-	$5x10^4$	-	-	-	7	189a	0
		B	-	$5x10^4$	-	-	-	7	189b	489
	190	T	+(FLOW)	-	$5x10^4$	-	-	7	190a	0
		T	+(FLOW)	-	$5x10^4$	-	-	7	190b	500
	191	T	-	-	$5x10^4$	-	-	7	191a	0
		T	-	-	$5x10^4$	-	-	7	191b	0
	192	B & T	+(FLOW)	$5x10^4$	$5x10^4$	-	-	7	192a	3825
		B & T	+(FLOW)	$5x10^4$	$5x10^4$	-	-	7	192b	4455

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.e.	193	B & T	-	$5x10^4$	$5x10^4$	-	-	7	193a	330
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	193b	495
	194	Unsep	+(FLOW)	-	-	10^5	-	7	194a	3224
		Unsep	+(FLOW)	-	-	10^5	-	7	194b	2542
	195	Unsep	-	-	-	10^5	-	7	195a	240
		Unsep	-	-	--	10^5	-	7	195b	280
	196	B & T	+	$5x10^4$	$5x10^4$	-	T800	7	196a	2925
		B & T	+	$5x10^4$	$5x10^4$	-	T800	7	196b	3000
	197	B & T	+	$5x10^4$	$5x10^4$	-	T800	7	197a	2600
		B & T	+	$5x10^4$	$5x10^4$	-	T800	7	197b	3160
	198	B & T	+	$5x10^4$	$5x10^4$	-	T1600	7	198a	2833
		B & T	+	$5x10^4$	$5x10^4$	-	T1600	7	198b	4862
	199	B & T	+	$5x10^4$	$5x10^4$	-	T1600	7	199a	4686

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
C.e.		B & T	+	$5x10^4$	$5x10^4$	-	T1600	7	199b	5808
	200	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	200a	8325
		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	200b	7659
	201	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	201a	7126
		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	201b	9990
	202	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	202	550
	203	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	203a	800
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	203b	650
R F.	204	B & T	+	$5x10^4$	$5x10^4$	-	T800	7	204a	733
		B & T	+	$5x10^4$	$5x10^4$	-	T800	7	204b	917
	205	B & T	+	$5x10^4$	$5x10^4$	-	T1600	7	205a	877
		B & T	+	$5x10^4$	$5x10^4$	-	T1600	7	205b	1055
	206	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	206a	1224

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.F.		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	206b	1440
	207	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	207a	117
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	207b	63
L.H.	208	B & T	+	$5x10^4$	$5x10^4$	-	T1000	7	208	1100
	209	B & T	+	$5x10^4$	$5x10^4$	-	T1000	7	209	1400
	210	B & T	+	$5x10^4$	$5x10^4$	-	T2000	7	210	1900
	211	B & T	+	$5x10^4$	$5x10^4$	-	T2000	7	211	1900
	212	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	212	3400
	213	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	213	3200
	214	B & T	++	$5x10^4$	$5x10^4$	-	T5000	7	214	2000
	215	B & T	+	$5x10^4$	$5x10^4$	-	T5000	7	215	3200
	216	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	216	3000
	217	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	217	1600

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
n.b.	218	Unsep	+	-	-	10^5	-	7	218a	5000
		Unsep	+	-	-	10^5	-	7	218b	8000
	219	Unsep	-	-	-	10^5	-	7	219	3750
	220	B	+	$5x10^4$	-	-	-	7	220a	350
		B	+	$5x10^4$	-	-	-	7	220b	250
	221	B	-	$5x10^4$	-	-	-	7	221a	300
		B	-	$5x10^4$	-	-	-	7	221b	200
	222	T	+	-	$5x10^4$	-	-	7	222a	200
		T	+	-	$5x10^4$	-	-	7	222b	0
	223	T	-	-	$5x10^4$	-	-	7	223a	200
		T	-	-	$5x10^4$	-	-	7	223b	0
	224	T	+	-	$5x10^4$	-	T3200	7	224a	0
		T	+	-	$5x10^4$	-	T3200	7	224b	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
n.b.	225	T	-	-	$5x10^4$	-	T3200	7	225a	200
		T	-	-	$5x10^4$	-	T3200	7	225b	200
	226	T	+	-	$5x10^4$	-	T6400	7	226a	200
		T	+	-	$5x10^4$	-	T6400	7	226b	200
	227	T	-	-	$5x10^4$	-	T6400	7	227a	0
		T	-	-	$5x10^4$	-	T6400	7	227b	0
	228	B & T	+	$5x10^4$	$5x10^4$	-	-	7	228a	4750
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	228b	6500
	229	B & T	-	$5x10^4$	$5x10^4$	-	-	7	229a	2800
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	229b	3800
	230	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	230	10600
	231	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	231a	6100
		B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	231b	8000

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
h.b.	232	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	232a	2500
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	232b	4800
	233	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	233a	1300
		B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	233b	600
k.e.	234	Unsep	+	-	-	10^5	-	7	234	7992
	235	Unsep	+	-	-	10^5	-	7	235	5500
	236	Unsep	-	-	-	10^5	-	7	236	900
	237	Unsep	-	-	-	10^5	-	7	237	1450
	238	B	+	10^5	-	-	-	7	238	800
	239	B	+	10^5	-	-	-	7	239	500
	240	B	+	10^5	-	-	-	7	240	700
	241	B	-	10^5	-	-	-	7	241	200
	242	T	+	-	$5x10^4$	-	-	7	242	200

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
K.e.	243	T	+	-	$5x10^4$	-	-	7	243	0
	244	T	-	-	$5x10^4$	-	-	7	244	0
	245	T	-	-	$5x10^4$	-	-	7	245	400
	246	T	+	-	$5x10^4$	-	T3200	7	246	200
	247	T	+	-	$5x10^4$	-	T3200	7	247	0
	248	T	-	-	$5x10^4$	-	T3200	7	248	0
	249	T	-	-	$5x10^4$	-	T3200	7	249	0
	250	T	+	-	$5x10^4$	-	T6400	7	250	0
	251	T	+	-	$5x10^4$	-	T6400	7	251	0
	252	T	-	-	$5x10^4$	-	T6400	7	252	0
	253	T	-	-	$5x10^4$	-	T6400	7	253	0
	254	B & T	+	$5x10^4$	$5x10^4$	-	-	7	254	2200
	255	B & T	+	$5x10^4$	$5x10^4$	-	-	7	255	1700

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
k.e.	256	B & T	-	$5x10^4$	$5x10^4$	-	-	7	256	500
	257	B & T	-	$5x10^4$	$5x10^4$	-	-	7	257	600
	258	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	258	20000
	259	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	259	12800
	260	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	260	5600
	261	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	261	6500
	262	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	262	1450
	263	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	263	1950
	264	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	264	2550
	265	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	265	1050
l.g.	266	Unsep	+	-	-	10^5	-	7	266	2100
	267	Unsep	+	-	-	10^5	-	7	267	1260
	268	Unsep	-	-	-	10^5	-	7	268	600

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PBL
l.g.	269	Unsep	-	-	-	10^5	-	7	269	1350	
	270	B	+	$5x10^4$	-	-	-	7	270	825	
	271	B	+	$5x10^4$	-	-	-	7	271	726	
	272	B	-	$5x10^4$	-	-	-	7	272	200	
	273	B	-	$5x10^4$	-	-	-	7	273	400	
	274	T	+	-	$5x10^4$	-	-	7	274	132	
	275	T	+	-	$5x10^4$	-	-	7	275	150	
	276	T	-	-	$5x10^4$	-	-	7	276	150	
	277	T	-	-	$5x10^4$	-	-	7	277	40	
	278	T	+	-	$5x10^4$	-	T3200	7	278	100	
	279	T	+	-	$5x10^4$	-	T3200	7	279	100	
	280	T	-	-	$5x10^4$	-	T3200	7	280	100	
	281	T	-	-	$5x10^4$	-	T3200	7	281	150	

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
I.g.	282	T	+	-	$5x10^4$	-	T6400	7	282	100
	283	T	+	-	$5x10^4$	-	T6400	7	283	100
	284	T	-	-	$5x10^4$	-	T6400	7	284	100
	285	T	-	-	$5x10^4$	-	T6400	7	285	50
	286	B & T	+	$5x10^4$	$5x10^4$	-	-	7	286	1060
	287	B & T	+	$5x10^4$	$5x10^4$	-	-	7	287	1600
	288	B & T	-	$5x10^4$	$5x10^4$	-	-	7	288	759
	289	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	289	8382
	290	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	290	10000
	291	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	291	350
	292	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	292	1100
	293	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	293	1450
	294	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	294	300

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
l.g.	295	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	295	900
d.b.	296	Unsep	+	-	-	10^5	-	7	296	2733
	297	Unsep	+	-	-	10^5	-	7	297	1500
	298	Unsep	-	-	-	10^5	-	7	298	2100
	299	Unsep	-	-	-	10^5	-	7	299	800
	300	B	+	$5x10^4$	-	-	-	7	300	300
	301	B	+	$5x10^4$	-	-	-	7	301	400
	302	B	-	$5x10^4$	-	-	-	7	302	200
	303	B	-	$5x10^4$	-	-	-	7	303	100
	304	T	+	-	$5x10^4$	-	-	7	304	0
	305	T	+	-	$5x10^4$	-	-	7	305	0
	306	T	-	-	$5x10^4$	-	-	7	306	0
	307	T	-	-	$5x10^4$	-	-	7	307	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
d.b.	308	T	+	-	$5x10^4$	-	T3200	7	308	0
	309	T	+	-	$5x10^4$	-	T3200	7	309	0
	310	T	-	-	$5x10^4$	-	T3200	7	310	0
	311	T	-	-	$5x10^4$	-	T3200	7	311	0
	312	T	+	-	$5x10^4$	-	T6400	7	312	0
	313	T	+	-	$5x10^4$	-	T6400	7	313	0
	314	T	-	-	$5x10^4$	-	T6400	7	314	0
	315	T	-	-	$5x10^4$	-	T6400	7	315	0
	316	B & T	+	$5x10^4$	$5x10^4$	-	-	7	316	2850
	317	B & T	+	$5x10^4$	$5x10^4$	-	-	7	317	2150
	318	B & T	-	$5x10^4$	$5x10^4$	-	-	7	318	1300
	319	B & T	-	$5x10^4$	$5x10^4$	-	-	7	319	700
	320	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	320	9000

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
d.b.	321	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	321	6800
	322	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	322	1400
	323	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	323	1500
	324	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	324	2800
	325	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	325	1600
	326	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	326	900
w.c.	327	Unsep	+	-	-	10^5	-	7	327	2000
	328	Unsep	+	-	-	10^5	-	7	328	1100
	329	Unsep	-	-	-	10^5	-	7	329	700
	330	Unsep	-	-	-	10^5	-	7	330	500
	331	B	+	$5x10^4$	-	-	-	7	331	400
	332	B	+	$5x10^4$	-	-	-	7	332	700
	333	B	-	$5x10^4$	-	-	-	7	333	200

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PBL
W.C.	334	B	-	$5x10^4$	-	-	-	7	334		400
	335	T	+	-	$5x10^4$	-	-	7	335		0
	336	T	+	-	$5x10^4$	-	-	7	336		0
	337	T	-	-	$5x10^4$	-	-	7	337		0
	338	T	-	-	$5x10^4$	-	-	7	338		0
	339	T	+	-	$5x10^4$	-	T3200	7	339		0
	340	T	+	-	$5x10^4$	-	T3200	7	340		0
	341	T	-	-	$5x10^4$	-	T3200	7	341		0
	342	T	-	-	$5x10^4$	-	T3200	7	342		0
	343	T	+	-	$5x10^4$	-	T6400	7	343		0
	344	T	+	-	$5x10^4$	-	T6400	7	344		0
	345	T	-	-	$5x10^4$	-	T6400	7	345		0
	346	T	-	-	$5x10^4$	-	T6400	7	346		0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
W.C.	347	B & T	+	$5x10^4$	$5x10^4$	-	-	7	347	1200
	348	B & T	+	$5x10^4$	$5x10^4$	-	-	7	348	1300
	349	B & T	-	$5x10^4$	$5x10^4$	-	-	7	349	400
	350	B & T	-	$5x10^4$	$5x10^4$	-	-	7	350	700
	351	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	351	4500
	352	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	352	2900
	353	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	353	900
	354	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	354	1000
	355	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	355	700
	356	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	356	400
	357	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	357	300
C.M.	358	Unsep	+	-	-	10^5	-	7	358	840
	359	Unsep	+	-	-	10^5	-	7	359	801

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PBL
c.m.	360	Unsep	-	-	-	10^5	-	7	360	479	
	361	Unsep	-	-	-	10^5	-	7	361	931	
	362	B	+	$5x10^4$	-	-	-	7	362	490	
	363	B	+	$5x10^4$	-	-	-	7	363	420	
	364	B	-	$5x10^4$	-	-	-	7	364	70	
	365	B	-	$5x10^4$	-	-	-	7	365	280	
	366	T	+	-	$5x10^4$	-	-	7	366	0	
	367	T	+	-	$5x10^4$	-	-	7	367	0	
	368	T	-	-	$5x10^4$	-	-	7	368	0	
	369	T	-	-	$5x10^4$	-	-	7	369	0	
	370	T	+	-	$5x10^4$	-	T3200	7	370	0	
	371	T	+	-	$5x10^4$	-	T3200	7	371	0	
	372	T	-	-	$5x10^4$	-	T3200	7	372	0	

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.m.	373	T	-	-	$5x10^4$	-	T3200	7	373	0
	374	T	+	-	$5x10^4$	-	T6400	7	374	0
	375	T	+	-	$5x10^4$	-	T6400	7	375	0
	376	T	-	-	$5x10^4$	-	T6400	7	376	0
	377	T	-	-	$5x10^4$	-	T6400	7	377	0
	378	B & T	+	$5x10^4$	$5x10^4$	-	-	7	378	1276
	379	B & T	+	$5x10^4$	$5x10^4$	-	-	7	379	1400
	380	B & T	-	$5x10^4$	$5x10^4$	-	-	7	380	373
	381	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	381	2520
	382	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	382	4690
	383	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	383	1330
	384	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	384	1524
	385	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	385	669
	386	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	386	560

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
c.m.	387	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	387	231
W.W.	388	B	+	$5x10^4$	-	-	-	7	388	363
	389	B	+	$5x10^4$	-	-	-	7	389	500
	390	T	+	-	$5x10^4$	-	-	7	390	400
	391	T	+	-	$5x10^4$	-	-	7	391	0
	392	T	-	-	$5x10^4$	-	-	7	392	100
	393	T	-	-	$5x10^4$	-	-	7	393	0
	394	T	+	-	$5x10^4$	-	T3200	7	394	0
	395	T	+	-	$5x10^4$	-	T3200	7	395	0
	396	T	-	-	$5x10^4$	-	T3200	7	396	0
	397	T	-	-	$5x10^4$	-	T3200	7	397	0
	398	T	+	-	$5x10^4$	-	T6400	7	398	0
	399	T	-	-	$5x10^4$	-	T6400	7	399	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
W.W.	400	T	-	-	$5x10^4$	-	T6400	7	400	0
	401	B & T	+	$5x10^4$	$5x10^4$	-	-	7	401	1600
	402	B & T	-	$5x10^4$	$5x10^4$	-	-	7	402	100
	403	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	403	3750
	404	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	404	5300
	405	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	405	1550
	406	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	406	1400
	407	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	407	100
d.s.	408	Unsep	+	-	-	10^5	-	7	408	1500
	409	Unsep	-	-	-	10^5	-	7	409	750
	410	B	+	$5x10^4$	-	-	-	7	410	700
	411	B	-	$5x10^4$	-	-	-	7	411	350
	412	T	+	-	$5x10^4$	-	-	7	412	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
d.s.	413	T	-	-	$5x10^4$	-	-	7	413	0
	414	T	+	-	$5x10^4$	-	T3200	7	414	0
	415	T	-	-	$5x10^4$	-	T3200	7	415	0
	416	T	+	-	$5x10^4$	-	T6400	7	416	0
	417	T	-	-	$5x10^4$	-	T6400	7	417	0
	418	B & T	+	$5x10^4$	$5x10^4$	-	-	7	418	1200
	419	B & T	-	$5x10^4$	$5x10^4$	-	-	7	419	800
	420	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	420	8600
	421	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	421	3800
	422	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	422	1550
	423	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	423	850
e.c.	424	B	+	$5x10^4$	-	-	-	7	424	550
	425	B	-	$5x10^4$	-	-	-	7	425	300

Subject	Culture No	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
e.c.	426	Unsep	+	-	-	10^5	-	7	426	1550
	427	Unsep	-	-	-	10^5	-	7	427	600
	428	B & T	+	$5x10^4$	$5x10^4$	-	-	7	428	1250
	429	B & T	-	$5x10^4$	$5x10^4$	-	-	7	429	550
	430	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	430	3700
	431	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	431	1000
	432	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	432	550
	433	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	433	300
j.h.	434	Unsep	+	-	-	10^5	-	7	434	2376
	435	Unsep	+	-	-	10^5	-	7	435	4000
	436	Unsep	+	-	-	10^5	-	7	436	4200
	437	Unsep	+	-	-	10^5	-	7	437	4800
	438	Unsep	-	-	-	10^5	-	7	438	825

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
j.h.	439	Unsep	-	-	-	10^5	-	7	439	900
	440	Unsep	-	-	-	10^5	-	7	440	500
	441	B	+	$5x10^4$	-	-	-	7	441	561
	442	B	+	$5x10^4$	-	-	-	7	442	726
	443	B	-	$5x10^4$	-	-	-	7	443	200
	444	B	-	$5x10^4$	-	-	-	7	444	0
	445	B & T	+	$5x10^4$	$5x10^4$	-	-	7	445	2342
	446	B & T	+	$5x10^4$	$5x10^4$	-	-	7	446	2640
	447	B & T	-	$5x10^4$	$5x10^4$	-	-	7	447	750
	448	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	448	7500
	449	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	449	10000
	450	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	450	2500
	451	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	451	2700

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
j.h.	452	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	452	3000
	453	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	453	330
	454	B	+	$5x10^4$	-	-	-	7	454	200
	455	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	-	7	455	6825
	456	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	-	7	456	8712
	457	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	-	7	457	2933
	458	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	-	7	458	2250
	459	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$ 3200	7	459	8844
	460	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$ 3200	7	460	8750
	461	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	$T\gamma^-$ 3200	7	461	2772
	462	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	$T\gamma^-$ 6400	7	462	2640
	463	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$ 6400	7	463	2800
	464	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$ 6400	7	464	3050

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
j.h.	465	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	$T\gamma^-$6400	7	465	800
j.d.	466	T	+	-	$5x10^4$	-	-	7	466	0
	467	T	+	-	$5x10^4$	-	-	7	467	0
	468	T	-	-	$5x10^4$	-	-	7	468	0
	469	T	-	-	$5x10^4$	-	-	7	469	0
	470	B	+	$5x10^4$	-	-	-	7	470	200
	471	B	-	$5x10^4$	-	-	-	7	471	50
	472	B & T	+	$5x10^4$	$5x10^4$	-	-	7	472	2175
	473	B & T	-	$5x10^4$	$5x10^4$	-	-	7	473	264
	474	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	-	7	474	4500
	475	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	-	7	475	6000
	476	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	-	7	476	2000
	477	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	-	7	477	1500

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
j.d.	478	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	478	3900
	479	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	479	4488
	480	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	480	600
	481	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$3200	7	481	6800
	482	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$3200	7	482	4500
	483	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	$T\gamma^-$3200	7	483	2000
	484	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	484	1040
	485	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	485	132
	486	B & $T\gamma$-	+	$5x10^4$	$5x10^4$	-	$T\gamma$-6400	7	486	1650
	487	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	$T\gamma^-$6400	7	487	1800
	488	B & $T\gamma^-$	-	$5x10^4$	$5x10^4$	-	$T\gamma^-$6400	7	488	500
	489	B & $T\gamma$-	-	$5x10^4$	$5x10^4$	-	$T\gamma^-$6400	7	489	400
m.m.	490	B	+	$5x10^4$	-	-	-	7	490a	284

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
M.M.		B	+	$5x10^4$	-	-	-	7	490b	142
	491	T	+	-	$5x10^4$	-	-	7	491a	0
		T	+	-	$5x10^4$	-	-	7	491b	0
	492	T	-	-	$5x10^4$	-	-	7	492a	0
		T	-	-	$5x10^4$	-	-	7	492b	0
	493	B & T	+	$5x10^4$	$5x10^4$	-	-	7	493a	3095
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	493b	3761
	494	B & T	-	$5x10^4$	$5x10^4$	-	-	7	494a	1999
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	494b	1143
	495	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	495a	8914
		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	495b	7542
	496	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	496a	2190
		B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	496b	3524

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
m.m.	497	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	497a	952
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	497b	1142
	498	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	498a	429
		B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	498b	571
b.w.	499	B	+	$5x10^4$	-	-	-	7	499	119
	500	B	+	$5x10^4$	-	-	-	7	500	95
	501	T	+	-	$5x10^4$	-	-	7	501	0
	502	T	+	-	$5x10^4$	-	-	7	502	0
	503	T	-	-	$5x10^4$	-	-	7	503	0
	504	B & T	+	$5x10^4$	$5x10^4$	-	-	7	504	1625
	505	B & T	+	$5x10^4$	$5x10^4$	-	-	7	505	1729
	506	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	506	3224
	507	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	507	2038

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
b.w.	508	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	508	629
e.j.	509	B	+	$5x10^4$	-	-	-	7?	509	71
	510	B	+	$5x10^4$	-	-	-	7	510	48
	511	T	+	-	$5x10^4$	-	-	7	511	0
	512	T	+	-	$5x10^4$	-	-	7	512	0
	513	T	-	-	$5x10^4$	-	-	7	513	0
	514	B & T	+	$5x10^4$	$5x10^4$	-	-	7	514	2061
	515	B & T	+	$5x10^4$	$5x10^4$	-	-	7	515	2429
	516	B & T	-	$5x10^4$	$5x10^4$	-	-	7	516	257
	517	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	517	2673
	518	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	518	1743
	519	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	519	429
M.W.	520	Unsep	+	-	-	10^5	-	7	520	2244

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PHL
M.M.	521	Unsep	+	-	-	10^5	-	7	521	4800	
	522	Unsep	-	-	-	10^5	-	7	522	700	
	523	Unsep	-	-	-	10^5	-	7	523	600	
	524	B	+	$5x10^4$	-	-	-	7	524	1600	
	525	B	+	$5x10^4$	-	-	-	7	525	1300	
	526	B	-	$5x10^4$	-	-	-	7	526	500	
	527	B	-	$5x10^4$	-	-	-	7	527	500	
	528	T	+	-	$5x10^4$	-	-	7	528	0	
	529	T	+	-	$5x10^4$	-	-	7	529	66	
	530	T	-	-	$5x10^4$	-	-	7	530	0	
	531	T	-	-	$5x10^4$	-	-	7	531	0	
	532	T	+	-	$5x10^4$	-	T3200	7	532	100	
	533	T	+	-	$5x10^4$	-	T3200	7	533	0	

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
[redacted]	534	T	-	-	$5x10^4$	-	T3200	7	534	0
	535	T	-	-	$5x10^4$	-	T3200	7	535	0
	536	T	+	-	$5x10^4$	-	T6400	7	536	0
	537	T	+	-	$5x10^4$	-	T6400	7	537	0
	538	T	-	-	$5x10^4$	-	T6400	7	538	0
	539	T	-	-	$5x10^4$	-	T6400	7	539	0
	540	B & T	+	$5x10^4$	$5x10^4$	-	-	7	540	5600
	541	B & T	+	$5x10^4$	$5x10^4$	-	-	7	541	7600
	542	B & T	-	$5x10^4$	$5x10^4$	-	-	7	542	800
	543	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	543	5500
	544	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	544	4500
	545	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	545	1500
	546	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	546	1600

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
M.W.	547	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	547	3200
	548	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	548	2300
	549	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	549	500
	550	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	550	500
J.F.	551	Unsep	+	-	-	10^5	-	7	551	600
	552	Unsep	+	-	-	10^5	-	7	552	400
	553	Unsep	-	-	-	10^5	-	7	553	300
	554	Unsep	-	-	-	10^5	-	7	554	200
	555	B	+	$5x10^4$	-	-	-	7	555	200
	556	B	+	$5x10^4$	-	-	-	7	556	200
	557	B	-	$5x10^4$	-	-	-	7	557	200
	558	B	-	$5x10^4$	-	-	-	7	558	100
	559	T	+	-	$5x10^4$	-	-	7	559	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
J.Y.	560	T	+	-	$5x10^4$	-	-	7	560	0
	561	T	-	-	$5x10^4$	-	-	7	561	0
	562	T	-	-	$5x10^4$	-	-	7	562	0
	563	T	+	-	$5x10^4$	-	T3200	7	563	0
	564	T	+	-	$5x10^4$	-	T3200	7	564	0
	565	T	-	-	$5x10^4$	-	T3200	7	565	0
	566	T	-	-	$5x10^4$	-	T3200	7	566	0
	567	T	+	-	$5x10^4$	-	T6400	7	567	0
	568	T	+	-	$5x10^4$	-	T6400	7	568	0
	569	T	-	-	$5x10^4$	-	T6400	7	569	0
	570	T	-	-	$5x10^4$	-	T6400	7	570	0
	571	B & T	+	$5x10^4$	$5x10^4$	-	-	7	571	800
	572	B & T	+	$5x10^4$	$5x10^4$	-	-	7	572	900

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
J.F.	573	B & T	-	$5x10^4$	$5x10^4$	-	-	7	573	400
	574	B & T	-	$5x10^4$	$5x10^4$	-	-	7	574	600
	575	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	575	300
	576	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	576	900
	577	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	577	400
	578	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	578	400
	579	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	579	100
	580	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	580	300
	581	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	581	300
	582	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	582	100
B.L.	583	Unsep	+	-	-	10^5	-	7	583	1000
	584	Unsep	+	-	-	10^5	-	7	584	1000
	585	Unsep	-	-	-	10^5	-	7	585	300

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PBL
B.L.	586	Unsep	-	-	-	10^5	-	7	586	800	
	587	B	+	$5x10^4$	-	-	-	7	587	300	
	588	B	+	$5x10^4$	-	-	-	7	588	100	
	589	B	-	$5x10^4$	-	-	-	7	589	200	
	590	B	-	$5x10^4$	-	-	-	7	590	100	
	591	T	+	-	$5x10^4$	-	-	7	591	0	
	592	T	+	-	$5x10^4$	-	-	7	592	0	
	593	T	-	-	$5x10^4$	-	-	7	593	0	
	594	T	-	-	$5x10^4$	-	-	7	594	0	
	595	T	+	-	$5x10^4$	-	T3200	7	595	0	
	596	T	+	-	$5x10^4$	-	T3200	7	596	0	
	597	T	-	-	$5x10^4$	-	T3200	7	597	0	
	598	T	-	-	$5x10^4$	-	T3200	7	598	0	

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.L.	599	T	+	-	$5x10^4$	-	T6400	7	599	0
	600	T	+	-	$5x10^4$	-	T6400	7	600	0
	601	T	-	-	$5x10^4$	-	T6400	7	601	0
	602	T	-	-	$5x10^4$	-	T6400	7	602	0
	603	B & T	+	$5x10^4$	$5x10^4$	-	-	7	603	900
	604	B & T	+	$5x10^4$	$5x10^4$	-	-	7	604	1500
	605	B & T	-	$5x10^4$	$5x10^4$	-	-	7	605	500
	606	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	606	1700
	607	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	607	1300
	608	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	608	400
	609	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	609	800
	610	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	610	500
	611	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	611	500

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.L.	612	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	612	500
B.F.	613	Unsep	+	-	-	10^5	-	7	613a	1400
		Unsep	+	-	-	10^5	-	7	613b	1700
	614	Unsep	-	-	-	10^5	-	7	614a	1000
		Unsep	-	-	-	10^5	-	7	614b	1300
	615	B	+	$5x10^4$	-	-	-	7	615a	300
		B	+	$5x10^4$	-	-	-	7	615b	300
	616	B	-	$5x10^4$	-	-	-	7	616a	100
		B	-	$5x10^4$	-	-	-	7	616b	100
	617	T	+	-	$5x10^4$	-	-	7	617a	0
		T	+	-	$5x10^4$	-	-	7	617b	0
	618	T	-	-	$5x10^4$	-	-	7	618a	0
		T	-	-	$5x10^4$	-	-	7	618b	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.F.	619	T	+	-	$5x10^4$	-	T3200	7	619a	0
		T	+	-	$5x10^4$	-	T3200	7	619b	0
	620	T	-	-	$5x10^4$	-	T3200	7	620a	0
		T	-	-	$5x10^4$	-	T3200	7	620b	0
	621	T	+	-	$5x10^4$	-	T6400	7	621a	0
		T	+	-	$5x10^4$	-	T6400	7	621b	0
	622	T	-	-	$5x10^4$	-	T6400	7	622a	0
		T	-	-	$5x10^4$	-	T6400	7	622b	0
	623	B & T	+	$5x10^4$	$5x10^4$	-	-	7	623a	1600
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	623b	1300
	624	B & T	-	$5x10^4$	$5x10^4$	-	-	7	624a	800
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	624b	600
	625	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	625a	1800

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.F.		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	625b	1600
	626	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	626a	900
		B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	626b	300
	627	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	627a	900
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	627b	800
	628	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	628a	800
		B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	628b	500
		B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	628c	300
H.M.	629	Unsep	+	-	-	10^5	-	7	629	1587
	630	Unsep	+	-	-	10^5	-	7	630	3571
	631	Unsep	-	-	-	10^5	-	7	631	1607
	632	Unsep	-	-	-	10^5	-	7	632	971
	633	B	-	$5x10^4$	-	-	-	7	633	144

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
H M	634	B	-	$5x10^4$	-	-	-	7	634	0
	635	T	+	-	$5x10^4$	-	-	7	635	0
	636	T	+	-	$5x10^4$	-	-	7	636	0
	637	T	-	-	$5x10^4$	-	-	7	637	0
	638	T	-	-	$5x10^4$	-	-	7	638	0
	639	T	+	-	$5x10^4$	-	T3200	7	639	0
	640	T	+	-	$5x10^4$	-	T3200	7	640	0
	641	T	-	-	$5x10^4$	-	T3200	7	641	0
	642	T	-	-	$5x10^4$	-	T3200	7	642	0
	643	T	+	-	$5x10^4$	-	T6400	7	643	0
	644	T	+	-	$5x10^4$	-	T6400	7	644	0
	645	T	-	-	$5x10^4$	-	T6400	7	645	0
	646	T	-	-	$5x10^4$	-	T6400	7	646	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
H.M.	647	B & T	+	$5x10^4$	$5x10^4$	-	-	7	647	2971
	648	B & T	+	$5x10^4$	$5x10^4$	-	-	7	648	1523
	649	B & T	-	$5x10^4$	$5x10^4$	-	-	7	649	143
	650	B & T	-	$5x10^4$	$5x10^4$	-	-	7	650	393
	651	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	651	2250
	652	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	652	2169
	653	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	653	997
	654	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	654	1679
	655	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	655	464
	656	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	656	857
F.B.	657	Unsep	+	-	-	10^5	-	7	657	1463
	658	Unsep	-	-	-	10^5	-	7	658	214
	659	Unsep	-	-	-	10^5	-	7	659	514

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
F.B.	660	B	+	$5x10^4$	-	-	-	7	660a	1429
		B	+	$5x10^4$	-	-	-	7	660b	571
	661	B	-	$5x10^4$	-	-	-	7	661a	429
		B	-	$5x10^4$	-	-	-	7	661b	286
	662	T	+	-	$5x10^4$	-	-	7	662	0
	663	T	+	-	$5x10^4$	-	-	7	663	0
	664	T	-	-	$5x10^4$	-	-	7	664	0
	665	T	-	-	$5x10^4$	-	-	7	665	0
	666	T	+	-	$5x10^4$	-	T3200	7	666	0
	667	T	+	-	$5x10^4$	-	T3200	7	667	0
	668	T	-	-	$5x10^4$	-	T3200	7	668	0
	669	T	-	-	$5x10^4$	-	T3200	7	669	0
	670	T	+	-	$5x10^4$	-	T6400	7	670	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
F B	671	T	+	-	$5x10^4$	-	T6400	7	671	0
	672	T	-	-	$5x10^4$	-	T6400	7	672	0
	673	T	-	-	$5x10^4$	-	T6400	7	673	0
	674	B & T	+	$5x10^4$	$5x10^4$	-	-	7	674	2829
	675	B & T	-	$5x10^4$	$5x10^4$	-	-	7	675a	1414
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	675b	1886
	676	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	676	2094
	677	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	677	3300
	678	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	678	286
	679	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	679	643
	680	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	680a	714
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	680b	571
	681	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	681	321

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
S.S.	682	Unsep	+	-	-	10^5	-	7	682	1232
	683	Unsep	-	-	-	10^5	-	7	683	429
	684	Unsep	-	-	-	10^5	-	7	684	349
	685	B	+	$5x10^4$	-	-	-	7	685a	330
		B	+	$5x10^4$	-	-	-	7	685b	809
	686	B	-	$5x10^4$	-	-	-	7	686	1000
	687	T	+	-	$5x10^4$	-	-	7	687	0
	688	T	+	-	$5x10^4$	-	-	7	688	0
	689	T	-	-	$5x10^4$	-	-	7	689	0
	690	T	-	-	$5x10^4$	-	-	7	670	0
	691	T	+	-	$5x10^4$	-	T3200	7	691	0
	692	T	+	-	$5x10^4$	-	T3200	7	692	0
	693	T	-	-	$5x10^4$	-	T3200	7	693	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Reds.	Culture duration	Plaque No.	PFC/10^6 PBL
S.S.	694	T	-	-	$5x10^4$	-	T3200	7	694	0
	695	T	+	-	$5x10^4$	-	T6400	7	695	0
	696	T	+	-	$5x10^4$	-	T6400	7	696	0
	697	T	-	-	$5x10^4$	-	T6400	7	697	0
	698	T	-	-	$5x10^4$	-	T6400	7	698	0
	699	B & T	+	$5x10^4$	$5x10^4$	-	-	7	699a	1107
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	699b	1357
	700	B & T	-	$5x10^4$	$5x10^4$	-	-	7	700a	446
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	700b	1250
	701	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	701	1071
	702	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	702	1423
	703	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	703	900
	704	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	704	520

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PEL
S.S.	705	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	705		456
	706	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	706		457
	707	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	707		400
L.H.	708	B	+	$5x10^4$	-	-	-	7	708		650
	709	B	+	$5x10^4$	-	-	-	7	709		858
	710	B	-	$5x10^4$	-	-	-	7	710		0
	711	B	-	$5x10^4$	-	-	-	7	711		300
	712	T	+	-	$5x10^4$	-	-	7	712		0
	713	T	+	-	$5x10^4$	-	-	7	713		100
	714	B & T	+	$5x10^4$	$5x10^4$	-	-	7	714		1419
	715	B & T	+	$5x10^4$	$5x10^4$	-	-	7	715		1122
	716	B & T	-	$5x10^4$	$5x10^4$	-	-	7	716		400
	717	B & T	-	$5x10^4$	$5x10^4$	-	-	7	717		100

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
L.H.	718	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	718	200
	719	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	719	100
	720	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	720	300
C.M.	721	T	+	-	$5x10^4$	-	-	7	721	0
	722	T	+	-	$5x10^4$	-	-	7	722	0
	723	T	-	-	$5x10^4$	-	-	7	723	0
	724	T	-	-	$5x10^4$	-	-	7	724	0
	725	B	+	$5x10^4$	-	-	-	7	725	300
	726	B	+	$5x10^4$	-	-	-	7	726	400
	727	B	-	$5x10^4$	-	-	-	7	727	100
	728	B	-	$5x10^4$	-	-	-	7	728	300
	729	B & T	+	$5x10^4$	$5x10^4$	-	-	7	729	1250
	730	B & T	+	$5x10^4$	$5x10^4$	-	-	7	730	1050

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6	PBL
C.M.	731	B & T	-	$5x10^4$	$5x10^4$	-	-	7	731	250	
	732	B & T	-	$5x10^4$	$5x10^4$	-	-	7	732	400	
D.B.	733	T	+	-	$5x10^5$	-	-	7	733a	0	
		T	+	-	$5x10^4$	-	-	7	733b	0	
	734	T	-	-	$5x10^4$	-	-	7	734a	0	
		T	-	-	$5x10^4$	-	-	7	734b	0	
	735	B	+	$5x10^4$	-	-	-	7	735a	650	
		B	+	$5x10^4$	-	-	-	7	735b	850	
	736	B	-	$5x10^4$	-	-	-	7	736a	100	
		B	-	$5x10^4$	-	-	-	7	736b	200	
	737	B & T	+	$5x10^4$	$5x10^4$	-	-	7	737a	3180	
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	737b	1740	
	738	B & T	-	$5x10^4$	$5x10^4$	-	-	7	738a	1250	

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
D.B.		B & T	-	$5x10^4$	$5x10^4$	-	-	7	738b	850
	739	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	739a	2800
		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	739b	3275
	740	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	740a	600
		B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	740b	1000
	741	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	741	3350
	742	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	742a	1950
		B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	742b	1150
S.W.	743	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	743	4362
	744	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	744	3075
	745	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	745	571
	746	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	746	571
	747	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	747	834

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
S.W.	748	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	748	1213
	749	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	749	572
	750	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	750	286
D.M.	751	T	+	-	$5x10^4$	-	-	7	751a	0
		T	+	-	$5x10^4$	-	-	7	751b	0
	752	T	-	-	$5x10^4$	-	-	7	752a	0
		T	-	-	$5x10^4$	-	-	7	752b	0
	753	B	+	$5x10^4$	-	-	-	7	753a	715
		B	+	$5x10^4$	-	-	-	7	753b	1287
	754	B	-	$5x10^4$	-	-	-	7	754a	286
		B	-	$5x10^4$	-	-	-	7	754b	0
	755	B & T	+	$5x10^4$	$5x10^4$	-	-	7	755a	2973
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	755b	3350

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
D. M.	756	B & T	-	$5x10^4$	$5x10^4$	-	-	7	756a	1793
		B & T	-	$5x10^4$	$5x10^4$	-	-	7	756b	944
	757	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	757a	3504
		B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	757b	4076
	758	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	758a	1716
		B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	758b	286
	759	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	759a	1144
		B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	759b	1001
	760	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	760a	286
		B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	760b	1287
N. B.	761	T	+	-	$5x10^4$	-	-	7	761a	0
		T	+	-	$5x10^4$	-	-	7	761b	0
	762	T	-	-	$5x10^4$	-	-	7	762a	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
N.B.		T	-	-	$5x10^4$	-	-	7	762b	0
	763	B	+	$5x10^4$	-	-	-	7	763a	114
		B	+	$5x10^4$	-	-	-	7	763b	86
	764	B & T	+	$5x10^4$	$5x10^4$	-	-	7	764a	1643
		B & T	+	$5x10^4$	$5x10^4$	-	-	7	764b	2679
	765	B & T	-	$5x10^4$	$5x10^4$	-	-	7	765	262
	766	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	766	1833
	767	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	767	257
B.L.	768	B	+	$5x10^4$	-	-	-	7	768	198
	769	B	+	$5x10^4$	-	-	-	7	769	200
	770	T	+	-	$5x10^4$	-	-	7	770	0
	771	T	-	-	$5x10^4$	-	-	7	771	0
	772	B & T	+	$5x10^4$	$5x10^4$	-	-	7	772	1687

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.L.	773	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	-	7	773	1765
	774	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	774	1665
	775	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	T3200	7	775	1775
	776	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	T6400	7	776	561
B.F.	777	T	+	-	$5x10^4$	-	-	7	777	0
	778	T	-	-	$5x10^4$	-	-	7	778	0
	779	$T\gamma^-$	+	-	$5x10^4$	-	$T\gamma^-$ 3200	7	779	0
	780	$T\gamma^-$	-	-	$5x10^4$	-	$T\gamma^-$ 3200	7	780	0
	781	B	+	$5x10^4$	-	-	-	7	781	280
	782	B	-	$5x10^4$	-	-	-	7	782	100
	783	B & T	+	$5x10^4$	$5x10^4$	-	-	7	783	1037
	784	B & T	-	$5x10^4$	$5x10^4$	-	-	7	784	473
	785	B & $T\gamma^-$	+	$5x10^4$	$5x10^4$	-	-	7	785	1332

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
B.F.	786	B & T_γ^-	-	$5x10^4$	$5x10^4$	-	-	7	786	475
	787	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	787	392
	788	B & T_γ^-	+	$5x10^4$	$5x10^4$	-	T_γ^- 3200	7	788	1450
	789	B & T_γ^-	-	$5x10^4$	$5x10^4$	-	T_γ^- 3200	7	789	996
	790	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	790	425
	791	B & T_γ^-	+	$5x10^4$	$5x10^4$	-	T_γ^- 3200	7	791	637
	792	B & T_γ^-	-	$5x10^4$	$5x10^4$	-	T_γ^- 6400	7	792	315
n.b.	793	B	+	$5x10^4$	-	-	-	7	793	250
	794	B	-	$5x10^4$	-	-	-	7	794	100
	795	T	+	-	$5x10^4$	-	-	7	795	0
	796	T	-	-	$5x10^4$	-	-	7	796	0
	797	B & T	+	$5x10^4$	$5x10^4$	-	-	7	797	4575
	798	B & T	-	$5x10^4$	$5x10^4$	-	-	7	798	2400

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
n.b.	799	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	799	8325
	800	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	800	3050
	801	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	801	5825
	802	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	802	2700
	803	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	803	5375
	804	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	804	2450
	805	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	805	5250
	806	B & T_4	-	$5x10^4$	$5x10^4$	-	-	7	806	2050
d.b.	807	B	+	$5x10^4$	-	-	-	7	807	425
	808	B	-	$5x10^4$	-	-	-	7	808	190
	809	T	+	-	$5x10^4$	-	-	7	809	0
	810	T	-	-	$5x10^4$	-	-	7	810	0
	811	B & T	+	$5x10^4$	$5x10^4$	-	-	7	811	2000

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
d.b.	812	B & T	-	$5x10^4$	$5x10^4$	-	-	7	812	800
	813	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	813	6700
	814	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	814	1500
	815	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	815	800
	816	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	816	390
	817	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	817	6880
	818	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	818	1332
	819	B & T_1	+	$5x10^4$	$5x10^4$	-	T3200	7	819	7200
	820	B & T_{11}	-	$5x10^4$	$5x10^4$	-	T3200	7	820	1100
	821	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	821	5100
	822	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	822	800
	823	B & T_2	+	$5x10^4$	$5x10^4$	-	T3200	7	823	5800
	824	B & T_2	-	$5x10^4$	$5x10^4$	-	T3200	7	824	1300

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
d.b.	825	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	825	4295
	826	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	826	600
	827	B & T_3	+	$5x10^4$	$5x10^4$	-	T3200	7	827	5150
	828	B & T_3	-	$5x10^4$	$5x10^4$	-	T3200	7	828	900
	829	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	829	1931
	830	B & T_4	-	$5x10^4$	$5x10^4$	-	-	7	830	800
	831	B & T_4	+	$5x10^4$	$5x10^4$	-	T3200	7	831	5300
	832	B & T_4	-	$5x10^4$	$5x10^4$	-	T3200	7	832	1300
k.e.	833	B	+	$5x10^4$	-	-	-	7	833	135
	834	T	+	-	$5x10^4$	-	-	7	834	0
	835	B & T	+	$5x10^4$	$5x10^4$	-	-	7	835	2204
	836	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	836	9049
	837	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	837	1220

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
k.e.	838	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	838	7472
	839	B & T_1	+	$5x10^4$	$5x10^4$	-	T3200	7	839	8859
	840	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	840	6637
	841	B & T_2	+	$5x10^4$	$5x10^4$	-	T3200	7	841	7759
	842	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	842	4320
	843	B & T_3	+	$5x10^4$	$5x10^4$	-	T3200	7	843	8118
	844	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	844	2028
	845	B & T_4	+	$5x10^4$	$5x10^4$	-	T3200	7	845	6608
M.W.	846	B	+	$5x10^4$	-	-	-	7	846	650
	847	B	-	$5x10^4$	-	-	-	7	847	150
	848	T	+	-	$5x10^4$	-	-	7	848	0
	849	T	-	-	$5x10^4$	-	-	7	849	0
	850	B & T	+	$5x10^4$	$5x10^4$	-	-	7	850	4600

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
M.W.	851	B & T	-	$5x10^4$	$5x10^4$	-	-	7	851	900
	852	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	852	3650
	853	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	853	1750
	854	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	854	3500
	855	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	855	800
	856	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	856	3250
	857	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	857	1000
S.S.	858	B	+	$5x10^4$	-	-	-	7	858	125
	859	B	-	$5x10^4$	-	-	-	7	859	0
	860	T	+	-	$5x10^4$	-	-	7	860	0
	861	T	-	-	$5x10^4$	-	-	7	861	0
	862	B & T	+	$5x10^4$	$5x10^4$	-	-	7	862	2171
	863	B & T	-	$5x10^4$	$5x10^4$	-	-	7	863	938

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
S.S.	864	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	864	2249
	865	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	865	688
	866	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	866	2281
	867	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	867	750
	868	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	868	2125
	869	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	869	563
	870	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	870	2495
	871	B & T_4	-	$5x10^4$	$5x10^4$	-	-	7	871	813
H.M.	872	B	+	$5x10^4$	-	-	-	7	872	286
	873	B	-	$5x10^4$	-	-	-	7	873	0
	874	T	+	-	$5x10^4$	-	-	7	874	0
	875	T	-	-	$5x10^4$	-	-	7	875	0
	876	B & T	+	$5x10^4$	$5x10^4$	-	-	7	876	2593

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
H.M.	877	B & T	-	$5x10^4$	$5x10^4$	-	-	7	877	1000
	878	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	878	2893
	879	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	879	1000
	880	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	880	2357
	881	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	881	929
	882	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	882	2460
	883	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	883	1071
	884	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	884	2404
	885	B & T_4	-	$5x10^4$	$5x10^4$	-	-	7	885	1500
H.B.	886	B	+	$5x10^4$	$5x10^4$	-	-	7	886	500
	887	B	-	$5x10^4$	$5x10^4$	-	-	7	887	100
	888	T	+	$5x10^4$	$5x10^4$	-	-	7	888	0
	889	T	-	$5x10^4$	$5x10^4$	-	-	7	889	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
H.B.	890	B & T	+	$5x10^4$	$5x10^4$	-	-	7	890	3048
	891	B & T	-	$5x10^4$	$5x10^4$	-	-	7	891	1400
	892	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	892	5200
	893	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	893	1050
	894	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	894	4650
	895	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	895	1100
	896	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	896	3550
	897	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	897	1100
	898	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	898	2950
	899	B & T_4	-	$5x10^4$	$5x10^4$	-	-	7	899	1050
D.B.	900	B	+	$5x10^4$	[illegible]	-	-	7	900	400
	901	B	-	$5x10^4$	-	-	-	7	901	200
	902	T	+	-	$5x10^4$	-	-	7	902	0

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
D.B.	903	T	-	-	$5x10^4$	-	-	7	903	0
	904	B & T	+	$5x10^4$	$5x10^4$	-	-	7	904	2500
	905	B & T	-	$5x10^4$	$5x10^4$	-	-	7	905	550
	906	B & T_1	+	$5x10^4$	$5x10^4$	-	-	7	906	2850
	907	B & T_1	-	$5x10^4$	$5x10^4$	-	-	7	907	600
	908	B & T_2	+	$5x10^4$	$5x10^4$	-	-	7	908	2425
	909	B & T_2	-	$5x10^4$	$5x10^4$	-	-	7	909	500
	910	B & T_3	+	$5x10^4$	$5x10^4$	-	-	7	910	2725
	911	B & T_3	-	$5x10^4$	$5x10^4$	-	-	7	911	550
	912	B & T_4	+	$5x10^4$	$5x10^4$	-	-	7	912	2200
	913	B & T_4	-	$5x10^4$	$5x10^4$	-	-	7	913	500
n.b.(B,T)	914	B	+	$5x10^4$	-	-	-	7	914	236
	915	B	-	$5x10^4$	-	-	-	7	915	143

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
n.b.	916	T	+	-	$5x10^4$	-	-	7	916	0
	917	T	-	-	$5x10^4$	-	-	7	917	0
	918	B & T	+	$5x10^4$	$5x10^4$	-	-	7	918	6257
	919	B & T	-	$5x10^4$	$5x10^4$	-	-	7	919	2429
	920	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	920	13766
	921	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	921	8357
	922	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	922	4857
	923	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	923	1571
D.M. (B) n.b. (T)	924	B & T	+	$5x10^4$	$5x10^4$	-	-	7	924	3829
	925	B & T	-	$5x10^4$	$5x10^4$	-	-	7	925	1571
	926	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	926	7571
	927	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	927	2357
	928	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	928	1579

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
D.M. (B) n.b. (T)	929	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	929	857
n.b. (B) D.M. (T)	930	B & T	+	$5x10^4$	$5x10^4$	-	-	7	930	2343
	931	B & T	-	$5x10^4$	$5x10^4$	-	-	7	931	536
	932	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	932	2874
	933	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	933	571
	934	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	934	1000
	935	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	935	214
m.m (B) n.b. (T)	936	B & T	+	$5x10^4$	$5x10^4$	-	-	7	936	3086
	937	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	937	11600
	938	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	938	1620
n.b. (B) m.m. (T)	939	B & T	+	$5x10^4$	$5x10^4$	-	-	7	939	3200
	940	B & T	-	$5x10^4$	$5x10^4$	-	-	7	940	1786
	941	B & T	+	$5x10^4$	$5x10^4$	-	T3200	7	941	7543

Subject	Culture No.	Cells cultured	PWM	B	T	Unsep.	Rads.	Culture duration	Plaque No.	PFC/10^6 PBL
n.b. (B) m.m. (T)	942	B & T	-	$5x10^4$	$5x10^4$	-	T3200	7	942	2714
	943	B & T	+	$5x10^4$	$5x10^4$	-	T6400	7	943	1464
	944	B & T	-	$5x10^4$	$5x10^4$	-	T6400	7	944	1000

APPENDIX 4 - OKT4, OKT5 ENUMERATION

Subject	Antibody or RPMI	% KILL AT VARYING MONOCLONAL ANTIBODY DILUTIONS							
		1/5	Mean%	1/10	Mean%	1/50	Mean%	1/100	Mean%
d.s.	OKT4	83/150, 85/166	168/316 53.1	88/164, 88/190	176/359 49	105/179, 85/135	190/314 60.5	72/192, 45/169	117/361 32.4
	OKT5	57/176, 60/189	117/355 32.9	24/83, 43/139	67/222 30.1	46/164, 29/109	75/273 27.4	40/185, 20/116	60/301 19.9
	RPMI	12/108, 9/89 14/129, 11/90	47/416 11						
a.c.	OKT4	57/95, 36/66	93/161 57.7	50/84, 38/79	88/163 53.9	50/107, 57/122	107/249 42.9	27/110, 17/119	44/229 19.2
	OKT5	25/107, 27/114	52/221 23.5	16/53, 23/69	39/122 31.9	9/46, 20/86	29/132 21.9	18/134, 20/104	38/238 15.9
	RPMI	12/108, 12/95	24/103 11.8						

SUBJECT	% KILL AT VARYING MONOCLONAL ANTIBODY DILUTIONS									
	RPMI	Mean%	OKT5 (1/10)	Mean%	OKT5 (1/50)	Mean%		OKT4 (1/50)	Mean%	
M.f.	12, 8 / 150 127	45/526	8.5	23, 29 / 115 119	76/434	17.5			67, 51 / 116 103	264/456	58
%Ty = 6	9, 16 / 100 149			12, 17 / 100 100					83 63 / 135 102		
K.e.	3, 0 / 109 103	3/212	1.4	18, 15 / 60 89	33/149	22.1			69, 50 / 149 138	119/187	41.4
c.m.	4, 0 / 800 0	4/800	0.5	22, 16 / 124 94	38/218	17			60, 50 / 131 114	110/245	45
%Ty = 13											
d.b.	5, 0 / 700 0	5/700	0.7	21, 23 / 106 106	68/402	0.7	12, 27 / 79 139	56/341	16	55, 33 / 136 103	173/468	37
%Ty =16				14, 10 / 94 96			17, 123			30, 55 / 98 131		
D.K.	12, 10 / 105 100	44/512	7	10, 13 / 100 100	43/300	14	15, 14 / 100 100	72/600	13	84, 58 / 143 120	247	52
%Ty =15	4, 4 / 107 100			20 / 100			16, 12 / 100 100			42, 63 / 105 109		
	6, 8 / 100 100						12, 9 / 100 100					

SUBJECT	% KILL AT VARYING MONOCLONAL ANTIBODY DILUTIONS											
	RPMI	Mean%		OKT5 (1/10)	Mean%		OKT5 (1/50)	Mean%		OKT4 (1/50)	Mean%	
M.L.	8/100, 2/100, 7/100, 4/100, 6/100, 3/100	30/600	5	23/108, 17/100, 10/100	50/308	16	8/100, 11/105, 6/100, 7/100	32/405	8	57/110, 62/103, 66/98, 61/100	246/411	60
C.S. ΣTγ= 11	19/116, 14/130, 20/130	53/376	14	42/104, 31/116, 62/164, 60/158	195/542	36	33/120, 30/150	63/270	23	55/87, 46/70, 75/123, 53/111	230/391	59
J.G. ΣTγ= 8	6/100, 8/100	14/200	7	25/66, 22/100	47/166	28				62/86	62/86	72
D.M. ΣTγ= 7	21/127, 15/130	36/157	14	63/187, 42/100, 31/125	136/412	33				80/117, 59/104, 68/106	207/327	63
M.K. ΣTγ= 10	30/219, 16/191, 14/142, 21/191	81/743	11	39/125, 22/124, 38/116, 38/108	137/473	30				46/98, 63/150, 62/120, 42/83	213/451	47

SUBJECT	% KILL AT VARYING MONOCLONAL ANTIBODY DILUTIONS																	
	RPMI		Mean%		OKT5 (1/10)		Mean%		OKT5 (1/50)		Mean%		OKT4 (1/50)		Mean%	
F.M. %Tγ=15	12, 119, 12, 100	18, 141	42/360	11	23, 88, 49, 157	46, 101, 29, 94	147/440	33					78, 137, 42, 81	82, 150, 46, 83	248/451	55
J.L. %Tγ=17	11, 139, 10, 113	9, 116, 10, 121	40/489	8	35, 96, 25, 98	46, 168, 22, 94	134/456	29	33, 151, 30, 174	37, 128, 22, 146	122/599	20	52, 106, 56, 110	54, 114, 41, 94	203/424	48
S.N. %Tγ=6	18, 149, 23, 151	18, 114, 10, 109	69/522	13	48, 166, 40, 120	38, 126, 28, 108	154/520	30					76, 120, 72, 107	89, 135, 90, 140	327/502	65
C.K. %Tγ=5	1, 100, 1, 200	1, 160, 0, 150	3/550	0.5	12, 100, 10, 100	10	32/300	11					67, 103, 63, 102	63, 102, 52, 99	245/406	60
E.M. %Tγ=13	19, 162, 9, 102	15, 202	43/466	9	25, 126, 18, 123	21, 128, 25, 141	89/518	17					53, 100, 66, 100	48, 102, 56, 111	223/413	54

SUBJECT	% KILL AT VARYING MONOCLONAL ANTIBODY DILUTIONS									
	RPMI	Mean%	OKT5 (1/10)	Mean%	OKT5 (1/50)	Mean%	OKT4 (1/50)	Mean%
A.T.	18/146, 25/159	43/305 14	35/113, 56/166	91/176 33			41/102, 50/105	91/207 44
R.M.	12/135, 8/118	21/253 8	22/100, 19/91	41/191 21			76/115, 79/147	155/262 59
S.N.	1/102, 8/176	9/278 3	11/111, 8/108	19/219 9			72/150, 50/142	122/292 42
T.M.	16/95, 14/103	30/198 15	20/144, 32/152	52/296 18			43/120, 44/85	87/205 42
	T γ DEPLETION STUDY							
K.e. %Tγ=0	3/100, 4/100, 4/100, 4/100, 1/100, 2/100, 0/100, 2/100, 0/100, 5/100, 2/100	27/1100 2	11/100, 5/100, 9/100	25/300 8	9/100, 10/100, 14/100	33/300 11	30/100, 54/138, 60/131	144/369 39

SUBJECT	RPMI	Mean%	% KILL AT VARYING MONOCLONAL ANTIBODY DILUTIONS OKT5 (1/10)	Mean%	OKT5 (1/50)	Mean%	OKT4 (1/50)	Mean%
	T_γ DEPLETION STUDY							
k.e. %Tγ=0	1/100 2/100 3/100; 0/100 2/100 1/100; 3/100 1/100 2/100; 4/100 3/100 1/100	23/1200 2	3/100 3/100; 5/100 5/100	16/400 4	4/100 2/100; 2/100 4/100	12/400 3	49/108 108/100; 49/103 69/121	210/432 49
c.e. %Tγ=12	1/100 3/100 3/100; 1/100 2/100 2/100; 2/100 2/100 5/100; 2/100 0/100 2/100	25/1200 2	9/100 15/100	24/200 12	9/100 8/100; 8/100 12/100	37/400 9	42/100 67/100; 58/130 50/110	197/440 45
c.e. %Tγ=0	1/100 0/100 0/100; 4/100 0/100 3/100; 1/100 2/100 3/100; 5/100 7/100	26/1100 2	1/100 2/100; 4/100 3/100	10/100 2.5	2/100 2/100; 2/100 3/100	9/400 2	57/120 55/107; 67/136 50/100	229/463 49

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