GAP JUNCTIONS IN LYMPHOCYTE ONTOGENY

Edward John Carolan

This Thesis is submitted for the degree of
Doctor of Philosophy
to The University of Glasgow

Faculty of Science
Institute of Biochemistry
University of Glasgow
University Avenue
GLASGOW G12 8QQ

December 1985

This Thesis is dedicated to the memory of my parents,
to Anne and William and Aunts Mary, Katherine and Theresa

TABLE OF CONTENTS

CHAPTER 2 cont'd ...

- o 0 o -

CHAPTER 3: RESULTS

CHAPTER 3 cont'd ...

CHAPTER 3 cont'd ...

- o 0 o -

- o 0 o -

- o 0 o -

LIST OF TABLES

LIST OF TABLES cont'd ...

LIST OF FIGURES

List of Abbreviations

Ab:	Antibody
ADA:	Adenosine deaminase
Ag:	Antigen
BSS:	Balanced Salt Solution
C:	Constant
CMC:	Complement mediated cytotoxicity
CON-A:	Concanavalin A.
CTL:	Cytotoxic T lymphocyte
CTL-p:	Cytotoxic T lymphocyte precursor
D:	Diversity
dAR:	Deoxyriboadenosine
dCR:	Deoxyribocytosine
dGR:	Deoxyriboguanosine
dTR:	Deoxyribothymidine
dUR:	Deoxyribouridine
DMEM:	Dulbecco's Modified Eagles Medium
DMSO:	Dimethyl Sulphoxide
DNA:	Deoxyribonucleic acid
DTH:	Delayed type hypersensitivity
EC:	Embryonal carcinoma
EDTA:	Ethylene diamine tetra-acetic acid
FACS:	Fluorescence activated cell sorter
FCS:	Foetal calf serum
FLS:	Forward light scatter
FTS:	Thymic serum factor
HAT:	Hypoxanthine: aminopterin: thymidine
HEPES:	Hydroxyethylene piperazine ethano-sulphonic acid
HGPRT:	Hypoxoanthine guanine phosphoribosyl transferase

HLA:	Human lymphocyte antigen
Hx:	Hypoxanthine
IFF:	Indirect immuno-fluorescence
Ig:	Immunoglobulin
IL-1:	Interleukin-1
IL-2:	Interleukin-2= T cell growth factor
IL-2R:	Interleukin-2 receptor
IMP:	Inosine monophosphate
J:	Joining
Lyt:	Lymphocyte T cell antigen
MEC:	Metabolic cooperation
MHC:	Major Histocompatability complex
NBCS:	New born calf serum
5'NT:	5' nucleotide
NV:	Non-viable
PBL:	Perferal blood lymphocyte
PBS:	Phosphate buffered saline
PEG:	Polyethylene glycol
PHA:	Phytohaemagglutinin
PNA:	Peanut agglutinin
PNP:	Purine nucleotide phosphatase
PPO:	Diphenyl oxazalone
RNA:	Ribonucleic acid
RPMI:	Roswell Park Memorial Institute
SCID:	Severe combined immuno-deficiency
SD:	Standard deviation
SF:	Serum free
T(c):	Cytotoxic T cell
T(DTH):	Delayed type hypersensitivity T cell

T(H): Helper T cell

T(Supp): Suppressor T cell

TCA: Trichloro-acetic acid

TdT: Terminal deoxnucleotidyl transferase

TK: Thymidine Kinase

TNC: Thymic nurse cell

TNF: Tumour necrosis factor

TPA: Tetradecanoyl phorbol acetate

TSF: Thymocyte stimulating factor

V: Variable

ZPA: Zone of polerizing activity

ACKNOWLEDGEMENTS

I would like to express my thanks to everyone who helped and supported me during the course of this work, especially my Supervisor, Dr J D Pitts, for his encouragement, advice and patience. I would also like to thank my Professorial Supervisor, Dr A R Williamson.

My special thanks go to Dr M E Finbow for helpful advice and discussions and also to the other members of the 'gap junction group', members of the Institute of Biochemistry and The Beatson Institute, for useful exchanges of ideas.

I am particularly indebted to Joanna Lee, for her consummate secretarial skills. Without her encouragement, dedication and sense of humour, this work would not have appeared in print. I also express my gratitude to Dr S Kimura for meticulous proof reading and to Prof H Festenstein for permitting me to complete my work for this Thesis.

I would also like to thank Drs D E Kipp, W Cushley and J Jones for providing monoclonal antibodies,the staff of the animal units of The Institute of Biochemistry and The Beatson Institute for supplying mice, the Wellcome Tissue Culture Unit and Mr H Scullion for operating the FACS. Without the help of these people the project could not have been completed.

This work was supported by The Cancer Research Campaign under Grant number SP1609.

SUMMARY

The primary aim of this research was to examine the ability of lymphoid cells to form permeable intercellular communicating junctions (gap junctions).

Established techniques, based on the detection of intercellular exchange of radiolabelled nucleotides, were used to analyse the junction formation between the adherent lymphoid tumour cell line RAJI and other cell lines of known junction forming ability. Results show that there is no detectable junction formation between RAJI cells, or between RAJI cells and any of the other cell types elaborately tested.

Peripheral blood lymphocytes (PBL) were examined, to resolve the conflict in the literature as to the ability of PBL to form communicating junctions. This required the development of modified procedures to analyse metabolite transfer from non-adherent cells. The results show no detectable junction formation between PBL and a range of junction-forming cell lines and the adherent lymphoid line RAJI. Phytohaemagglutinin (PHA) treated PBL also show no detectable junction formation between the same range of cells, which is in conflict with reported electrophysiological findings.

Attempts were made to improve the sensitivity of the assay system used for junction formation, by fixation of the acid soluble component of the incorporated radiolabelled material. Retention of the label is increased, but the increase was not sufficient to significantly improve the assay.

The analysis was extended to immature lymphocytes of the thymus, and population of thymocytes was identified which was able to form junctions with murine fibroblasts, Swiss 3T3 and a BALB/c 3T3 and

the hypoxanthine guanine phosphoribosyl transferase mutant ($HGPRT^-$) subline of BHK/C13 baby hampster kidney fibroblast line TG2.

Further analysis demonstrates that the size of this population varies with the age of the mouse. Isopycnic centrifugation shows that this population is proportionately higher in low density fractions.

Peanut agglutinin (PNA) fractionation shows that the thymocytes which can form junctions can also be agglutinated with PNA.

Thymocytes were fractionated using a range of monoclonal antibodies specific for a range of lymphocyte surface antigens using complement mediated cytotoxicity (CMC) and fluorescence activated cell sorting (FACS).

These experiments show that the thymocytes which can form junctions lack expression of Lyt-2 and Ia antigens, but express Lyt-1 molecules. However, there is variability in the expression of Thy-1 and H-2K antigens and the frequency of thymocytes that can form junctions is of the order of $5x10^{-4}$ and 10^{-5} cells.

The implications of the immature surface antigen expression and the ability to form junctions are discussed and the position of thymocytes which can form junctions in intrathymic lymphocyte differentiation and development are explored.

CHAPTER 1

INTRODUCTION

'Sehen ist eine schwere Kunst'

('To see is a difficult art')

Schleiden c1800

1:1 Intercellular Communication

Complex multicellular organisms have evolved by varied cellular differentiation into many forms. Such differentiation has led to a division of labour between component cells and has produced organisms which, at a cell functional level, are phenotypic mosaics. Organism function depends on phenotypically different cells acting in concert and such co-ordination in turn depends on intercellular communication within and between cell phenotypes.

As evolution has progressed, methods of intercellular communication must have co-evolved. Those methods of which we now know, can be divided into two types depending whether they utilise an extracellular signalling pathway or an intracellular pathway. Communication by the extracellular pathway is characterised by signal substances which are secreted across or inserted into the outer surface of the cytoplasmic membrane of one cell and the binding of signal substance to a receptor on or in the target cell. The binding of the signal molecule produces a phenotypic modification in the target cell. The initiating and target cells may be adjacent, as in neurotransmission at the chemical synapse, or widely separated, as in hormonal control of sexual development. Interactions of membrane bound signal and receptor molecules are exemplified by membrane/membrane interactions of lymphoid cells and dendritic or antigen presenting cells in the generation of immune responses.

The other pathway of intercellular communication allows cytosol/cytosol interactions via specialised transmembrane channels known as gap junctions.

1:2 Cytoplasmic Coupling

Short range intercytoplasmic coupling was first observed by Furshpan and Potter (1959) when examining electrical potentials in

crayfish neurones. A current pulse introduced into one cell passed into the contiguous adjacent cell. From the short transmission time of the current pulse and the low electrical resistance, 10^4 times lower than normal cytoplasmic membranes, they proposed that there existed some sort of cytoplasmic continuity between cells that were electrically coupled. Initially it was thought that these low resistance pathways were restricted to certain excitable cells. However, Loewenstein and Kanno (1964) and Furshpan and Potter (1968) discovered that exactly similar communication occurred between salivary gland cells of Drosophila, as well as between cells in embryos and in tissue culture. Furthermore, when low molecular weight fluorescent dyes, to which the cytoplasmic membrane was relatively impermeable, were injected into single salivary gland cells, the dye passed into contiguous cells.

Prior to the discovery of the low resistance pathways, it was observed by Robertson (1963) that contiguous cells in thin-section electron microscopy possessed intramembraneous structures where the adjacent membranes were separated by only a 2-4nm gap. These structures, which were termed gap junctions, have been further characterised, by other groups notably Revel and Karnovsky (1967) and Benedetti and Emmlot (1968) by electron microscopy and extensive correlative evidence indicates that these structures are the source of cytoplasmic continuity in coupled cells (Loewenstein, 1979; Finbow, 1982).

Independently, an accidental observation by Subak-Sharpe, Burk and Pitts in 1966, provided another method of detecting and quantifying gap junction mediated cell coupling. They observed that certain mutant animal cells growing in culture lost their mutant phenotype when grown in contact with wild type cells. The initial experiments

were performed with a cell line in which the hypoxanthine:guanine phosphoribosyl transferase (HGPRT) enzyme was defective resulting in an inability to incorporate [^{3}H]-hypoxanthine into nucleic acid. However, when the cell line, in the presence of [^{3}H]-hypoxanthine, was co-cultured with wild type cells (HGPRT$^+$) labelled material was found in the nucleic acid of mutant cells in contact with wild type cells. This phenomenon was called metabolic co-operation (MEC) and was more graphically demonstrated in a series of experiments using HGPRT$^-$ cells and Thymidine Kinase (TK$^-$) cells in the presence of aminopterin which inhibits the de novo synthesis of purine nucleotides and dTMP. With aminopterin, even in the presence of exogenous hypoxanthine and thymidine, neither the HGPRT$^-$ nor the TK$^-$ cells can grow, but in mixed cultures the HGPRT$^-$ cells synthesis dTMP and TK$^-$ cells produce purine nucleotides for both cell types. The cultures survive only if they are confluent, if both cell types are capable of communication by gap junction formation, and if sufficient of each phenotype is present (Pitts, 1972).

With the mutant cell technique, and an extension of this method using cells prelabelled with [^{3}H]-uridine (Pitts and Simms, 1977) it has been possible to assess the cytoplasmic coupling potential of many cell types. Prelabelled cells (donors) which, after washing, contain [^{3}H]-uridine nucleotides and [^{3}H]-RNA, can be added to unlabelled cells (recipients) and after co-culture, processed for autoradiagraphy. By counting the number of silver grains over recipients in contact with labelled donors and over cells not in contact with donors, a measure of junctional communication can be estimated.

From these initial observations three basic methods have evolved for the detection and quantitation of cytoplasmic coupling,

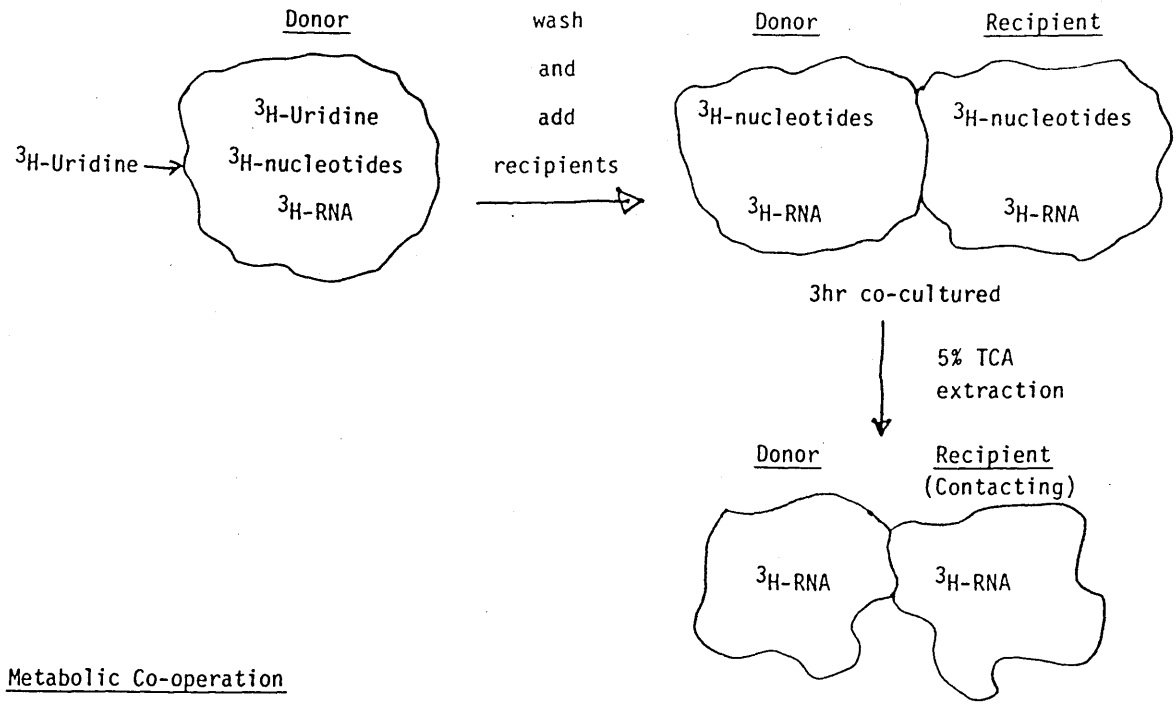
electrophysiology, dye transfer and radio-labelled metabolite exchange. Each technique has its own strengths and weaknesses both in practical limitations and sensitivity. Insertion of microelectrodes into cells, inducing current pulses and the monitoring of potential differences in other impaled cells has the advantage of great sensitivity and quantitative accuracy in defining cytoplasmic coupling between cells. However, the technique requires high technical skill, sophisticated apparatus and complex analysis if performed on tissues or large cell sheets, where if the current pulse is injected into a cell distant from the recording or monitoring electrode, the pulse will spread radially through the coupled cells and the signal will be attenuated.

As fluorescent dye injection, usually by iontophoresis, utilises the same apparatus, it also requires high technical skill to impale the cell and the progress of the dye can only be monitored by fluorescence microscopy. The quanitity of fluorescence transferred can be quanitified using photodetectors and transfer rates can be estimated. However, dye transfer differs from electrophysiology in the fact that the injected dye is 'foreign' whereas the alterations induced electrophysiologically are closer to normal electro-physiological and biochemical conditions. However, both these techniques have contributed enormously to the understanding of cytoplasmic coupling both in studies on rates of formation and transfer and in estimating the molecular weight limits of molecules which can be transferred and thus the size restrictions of the junctional channels themselves (See Section 1:3).

The other method of detecting and quantitating cytoplasmic coupling is labelled metabolite exchange. This method has the advantage that the labelled substances are normal cell components, but it is dependent on the metabolic state of the cells involved.

Figure 1

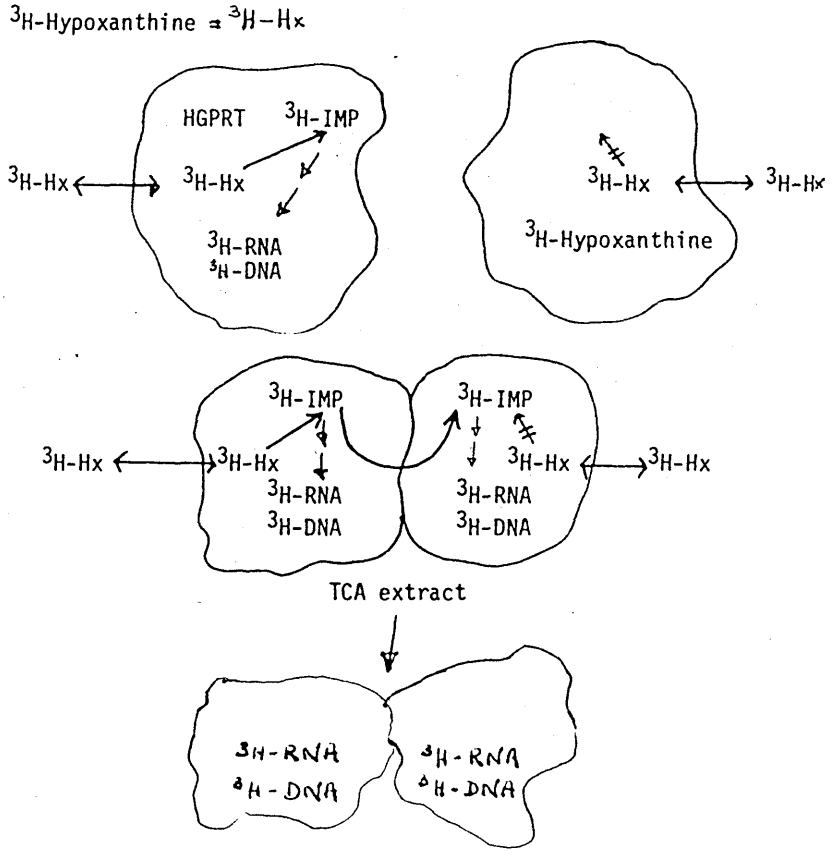
URIDINE NUCLEOTIDE TRANSFER



Metabolic Co-operation

Wild Type

Mutant (HGPRT$^-$) or TK$^-$



It is a quantitative, reproducible and simple technique but is limited by the length of time required for the autoradiographic process. From the measurements of radioactivity in different cell fractions (acid soluble and acid insoluble) total incorporation, intercellular pool sizes, mean cellular incorporation and the distributions of cellular incorporation can be quanitified and assess the ability of a particular cell type to act as a donor or recipient. The principles of both uridine nucleotide transfer and metabolic cooperation are shown in Figure 1. From this diagram it is evident that the metabolic state of the cell pair involved in junction formation is an important factor in the detection of junction formation by nucleotide exchange. Only in cell pairs where the donor is metabolically active and the recipient cell is metabolically inactive would an underestimate of metabolite exchange result. However, this situation can be experimentally overcome by using each cell type as donor and recipient in combination with characterised cell types (eg cells of established lines).

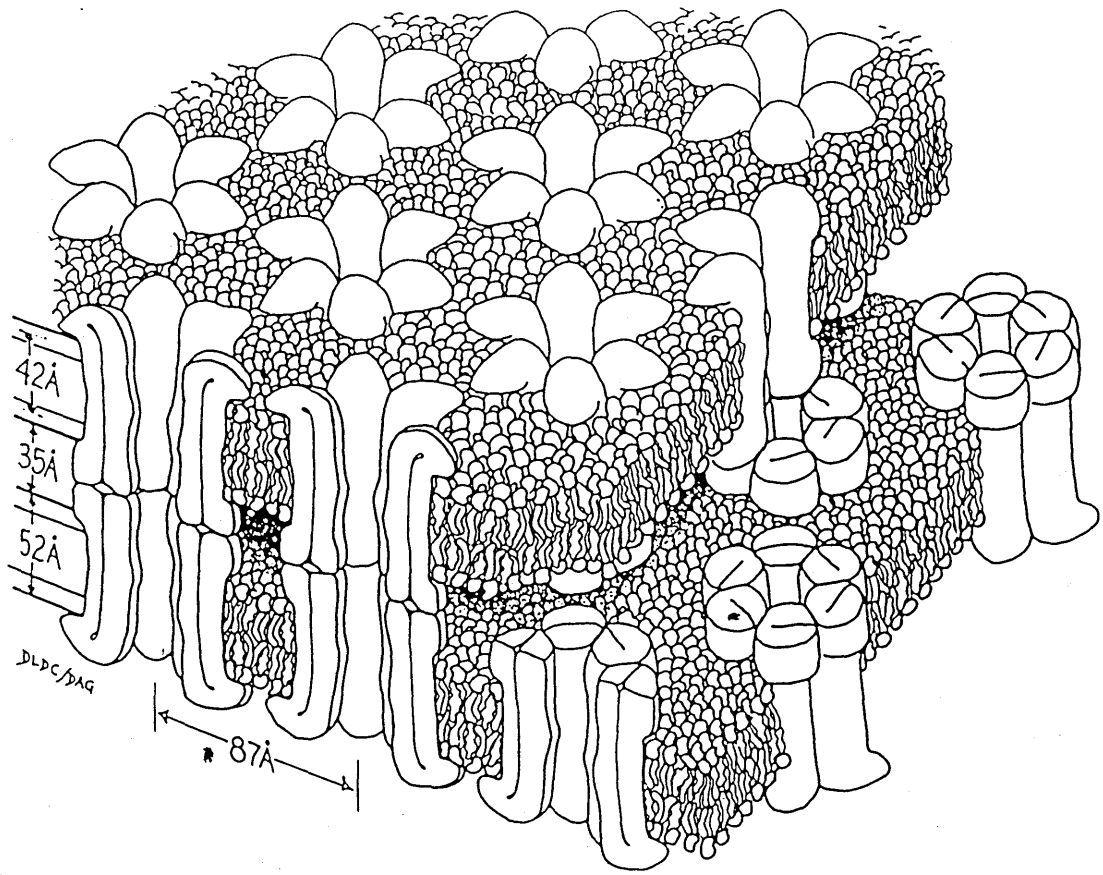
Using these different techniques cytoplasmic coupling has been demonstrated between cells from jellyfish and through all metazoon animals to arthropods and man. Very few types of animal cells do not form gap junctions and these are restricted to skeletal muscle, some nerve cells and circulating blood cells.

1:3 Morphology and Structure of Gap Junctions

In thin-section transmission electron microscopy, gap junctions appear as regions of close apposition between adjacent cells where the total width of both the bi-layers and the reduced intercellular space is approximately 17nm. Between the apposed bi-layers there is a 2-4nm 'gap' into which transmembranous particles project and

Figure 2

DIAGRAMATIC REPRESENTATION OF GAP JUNCTION STRUCTURE



Goodenough D.A.

meet (Revel and Karnovsky, 1967). When sections are made after infiltration with lanthanum hydroxide the gap is delineated more clearly and the apposed areas reveal a pentilaminar structure. Uranyl acetate, on the other hand, reveals a septilaminar structure due to the staining of the bi-layers. In oblique en face thin section electron microscopy, areas filled with hexagonal arrays of what appears to be annuli, with centre to centre spacing of 9nm, in a plane parallel to the membrane surface can occasionally be seen. These annuli have an external diameter of 8nm (Revel and Karnovsky, 1967).

In freeze fracture electron microscopy, the areas of membrane apposition appear as plaques of particles. On fracture, the plane of cleavage passes between the bi-layer leaflets revealing hexagonal arrays of protusions on the protoplasmic face (in vertebrate tissues) and corresponding patterns of indentations on the endoplasmic face (McNutt and Weinstein, 1970). The particles or 'connexons' (Goodenough and Gilula, 1974) are 8nm in diameter with a central depression of 2nm occasionally seen.

A structure has been proposed by correlating electron microscopy and X-ray diffraction, (Casper et al, 1977; Makowski et al, 1977) obtained from pellets of isolated junctional plaques, showing long range order, but short range disorder. Electron diffraction analysis also shows a regular hexagonal packing, six-fold symmetry and a central pore (Unwin and Zampighi, 1980). A representation of the structure is shown in Figure 2.

The constituent protein of gap junctional plaques can be isolated from plasma membrane preparations of tissue where the cells are known to be cytoplasmically coupled. Initial experiments with junctional plaques showed equal amounts of protein and phospholipid with the phospholipid component being similar to that of the non-junctional

plasma membrane and the protein constituent to have no detectable carbohydrate (Culvenor and Evans, 1977). The extraction procedure from crude plasma membrane can be monitored by electron microscopy using the characteristic morphology of the junctional plaques. However, there has been considerable debate concerning the molecular weight of the junctional protein (Finbow et al, 1983).

Initially, plasma membrane preparations from heart or liver were used because these tissues give unusually high yields of plasma membranes and these were treated with proteases prior to membrane extraction with detergent. These preparations contained one or two protein components with a molecular weight of 10,000-12,000 and contained morphologically pure junctional plaques (Goodenough and Stockenius, 1972; Goodenough and Gilula, 1974; Goodenough, 1976). However, more recent studies using denaturing agents rather than proteases to remove contaminants have yielded a molecular weight of 26,000-28,000 (Herzberg and Gilula, 1979; Henderson et al, 1979; Finbow et al, 1980).

More recent work has demonstrated that the junctional subunit has a molecular weight of 16,000 using a method which will extract junctional protein not only from animal tissue, but from tissue culture cell grown in vitro. This method utilizes an initial detergent (Triton) extraction step and like earlier methods depends subsequently on the ability of junctional plaques to resist denaturing agents (6M urea, sarkosyl) and proteases (Trypsin) (Finbow et al, 1983).

1:4 Permeability of Junctional Channels

The presence of gap junctions between cells has been correlated (Gilula et al, 1972) with the exchange of ions (electrical coupling) and small molecules (dye coupling) and metabolite transfer. Ion

transfer is believed to be responsible for the changes in potential difference in contiguous cells after current pulse injection (Furschpan and Potter, 1959; Loewenstein and Kanno, 1964; Sheridan et al, 1978), the acquisition of synchronous beating in cultured myocardial cells (Griepp and Bernfield, 1978) and the survival of ouabain sensitive primate cells when co-cultured with resistant rodent cells in the presence of the Na^+/K^+ATPase inhibitor ouabain(Pitts and Shaw,1981).

A variety of fluorescent dyes have been used to prove the molecular weight exclusion limit of junctional transfer (Loewenstein and Kanno, 1964; Johnson and Sheridan, 1971) with Chicago sky blue (Mr 993) being the highest molecular weight that has passed into coupled cells after micro-injection (Potter et al, 1966).

Nucleotide transfer has been demonstrated (Cox et al, 1976; Pitts , 1971) but not the transfer of proteins, RNA or DNA (Pitts and Simms, 1977). In similar experiments Finbow and Pitts (1981) demonstrated that sugars, sugar phosphates, choline phosphates, CDP-choline, proline or its precursors, lower glutamated forms of tetrahydrofolate, but not tetraglutamated tetrahydrofolate (Mr 960) or phospholipids were exchanged between coupled cells.

In experiments using mouse myocardial cells and rat granulosa cells, the transfer of cyclic AMP (cAMP) was demonstrated. Myocardial cells and granulosa cells react to catacholamines and follicle stimulating hormone (FSH) respectively by producing cAMP intracellularly, which acts as a second messenger resulting in an increase in myocardial cell beating rate and the production of plasminogen activator in the granulosa cells. Neither cell is sensitive to the wrong hormone, but when grown together in confluent culture either FSH or noradrenaline will elicit both phenotypic

alterations in all coupled cells, presumably by the transfer of the second messenger, cAMP, through gap junction channels (Lawrence et al, 1978).

The size limit of molecules which can pass through gap junction channels has been estimated at about Mr 1,000 for mammals (Pitts and Simms, 1977; Flagg-Newton et al, 1979; Finbow and Pitts, 1981) and Mr 1,500 for arthropods (Simpson et al, 1977). Mr is quoted as it is a convenient but incomplete indication of molecular size. The hydrated diameter of transferred molecules would be a better indicator as to the size restrictions of junctional channel, but in general these dimensions are not known.

1:5 The Function of Gap Junction Mediated Intercellular Communication

At the 2 and 4 cell stages of the early mouse embryo there is no gap junction mediated coupling (Goodall and Johnson, 1982) but from the 8 cell state electrical and dye coupling are observed between all cells (Lo and Gilula, 1979a). However, early reports led to confusion as electrical coupling and dye transfer in the early cleavage stages was observed. The problems which gave rise to this confusion was that large areas of cell-cell contact may give rise to spurious electrical coupling and residual cytoplasmic bridges, which are sometimes hard to detect morphologically, give rise to ion and dye transfer which may incorrectly be attributed to gap junction mediated intercellular communication. Using horseradish peroxidase to detect residual cytoplasmic bridges, electrophysiology, dye injection and electron microscopy, on the early mouse embryo, junctions are first observed in the early compaction 8 cell stage embryo (Dulcibella et al, 1975; Magnuson et al, 1978; Lo and Gilula, 1979a). Electrical and dye coupling, which are only seen in sister blastomeres in the

earlier embryo, is accompanied by enzyme transfer indicating the presence of cytoplasmic bridges. At compaction, injected dye passes into all 8 cells but enzyme transfer is only seen to a single sister blastomere indicating the presence of residual cytoplasmic bridges. Throughout compaction and blastocyst development junctional communication is retained, and dye injected into the pre-implantation blastocyst trophoblast cell spreads throughout the entire embryo including the inner cell mass (Lo and Gilula, 1979a).

In the post-implantation embryo, dye injected into a trophoblast cell spreads throughout the trophoblast cells but not to adjacent inner cell mass cells and vice versa. Ionic coupling however, is maintained between trophoblast and the inner cell mass, which probably reflects the increased sensitivity of electrophysiology over dye transfer rather than indicating differential permeability. Further restriction of dye transfer is observed in inner cell mass cells of more developed embryos, suggesting that further compartmentation, probably of this incomplete kind, is occurring (Lo and Gilula, 1979b).

The presence of electrical coupling in the absence of dye transfer is seen between insect cuticle epithelium at segmental boundaries. Each segment of cuticle epithelium is thought to be a separate developmental compartment and morphologically identifiable gap junctions are observed between cells in individual segments and between adjacent segments. However, dye injected into an epithelial cell of a segment will pass to all other cells in the segment but not across the segmental boundary, although the segments are electrically coupled. This observation is again probably due to the differences in the sensitivity of the two methods of measuring junction mediated coupling, but whether this difference in junctional transfer has any significance in development remains to be demonstrated (Warner

and Lawrence, 1973). However, the appearance of junctional coupling in the mouse embryo, at the 8 cell stage, precedes the 16-32 cell stage, when determination is believed to first occur (Gardner and Rossant, 1976).

Using embryonal carcinoma (EC) cells, which are now thought of as embryonic cells showing malignant properties due to abnormal microenvironment (Graham, 1977) and cells from early mouse embryos, it was found that metabolic co-operation occurred between EC cells and cells from the morula, the inner cell mass of the blastocyst, endoderm, mesoderm and ectoderm of the eighth day egg cylinder, but not the trophectoderm or its derivatives (Gaunt and Papioannou, 1979). As EC cells are closely related to the inner cell mass and embryonic ectodermal cells (Graham, 1977) this adds weight to the observations of Lo and Gilula (1979b) that these cells become metabolically uncoupled, while retaining electrical coupling, from the trophectoderm during normal embryonic development.

Embryonic induction has been followed in vitro by prelabelling inducing tissues with ^{3}H-uridine and following the movement of labelled material, by electron microscope autoradiography to the induced tissue in toad gastrulae. The earliest incorporation in the induced tissue was seen in the nucleus, particularly the nucleoli. This was initially interpreted as showing RNA transfer or nucleotide transfer (Kelley, 1968). However, subsequent work has demonstrated this transfer of label from mesenchyme to epithelium to be junction mediated nucleotide transfer (Grainger and Wessels, 1974). In explants of mouse embryonic tissue it has been shown that the induction process leading to mouse kidney tubule formation can be correlated with close approaches and cell contact between the cells of the interacting tissue (Wartiovarra

et al, 1972). Another, if somewhat tangential, observation that implicates the role of junctional communication in development has been that retinoic acid at 10^{-4}M has been shown to uncouple junctional communication measured by tritiated nucleotide transfer (Pitts et al, manuscript in preparation). Retinoic acid also causes EC cell differentiation (Jetten and Jetten, 1979), epithelial cell differentiation (Lotan, 1980), reduplication in axolotl limb regeneration (Maden, 1982) and replaces the zone of polarizing activity (ZPA) in experiments on chick limb development (Tickle et al, 1982). Whether or not retinoic acid altering gap junction mediated intercellular communication in vitro can be related to these phenomena or for that matter, the role of gap junctions in the development of the organism, both remain to be proved.

The role of gap junctions in excitable tissue is much more clearly understood. There has been considerable work on electrotonic synapses between neurones, where speed of response is important. Furshpan and Potter (1959) discovered that pre- and post-synaptic fibres of the giant motor synapses of the crayfish are coupled ionically and that the junctions acted as rectifiers allowing transmission only in one direction. An advantage, in tissues like heart or smooth muscle, of transmission of action potentials from cell to cell, via gap junctions, is that the wave of depolarisation can pass through junctional channels into all surrounding coupled cells and thus spread radially from the source of the initiation. This results in the acquisition of synchronous beating in myocardial cells in vitro (Griepp and Bernfield, 1978). Furthermore, in vivo studies have also shown that the atrioventricular node shows substantially reduced areas of gap junctions, which is consistant with this structure introducing

a delay in the transmission of the contraction signal between the atrium and ventricle (Pollack, 1976).

Retinal cells are thought to use gap junctional communication as a means of regulating visual acuity in response to levels of illumination. It has been proposed that the retinal cells, by means of their junctions, disperse electrical 'background noise' to surrounding cells and thus dissipate unwanted signals before transmission to the visual cortex.

The only reported incidence of the induction of gap junctions is in an excitable tissue, the myometrium of the pre-natal uterus. The myometrium of the normal, pregnant and post partum uterus has the small junctional plaques commonly found between smooth muscle cells. However, prior to parturition, the myometrial junctional plaque size and number of plaques per cell increases almost 200-fold (Garfield et al, 1977; 1978). It has been suggested that gap junction formation on this scale could function to co-ordinate and facilitate uterine contractions at birth.

In the adult, in addition to the coupling of excitable cells, gap junction formation may give rise to synchronous germ cell development in the testis by coupling of the sertoli cells of the seminiferous tubules (Gilula et al, 1976). Junctional communication between oocytes and granulosa cells may play a role in the metabolic support of the developing oocyte (Moor et al, 1980) and may be necessary for the maintenance of meiotic arrest during the development of the oocyte (Anderson and Albertini, 1976). Moor et al (1980) discovered that in the oocyte/cumulus cell complex, the oocyte could incorporate [^{3}H]-uridine and [^{3}H]-choline as well as the cumulus cells on their own, but oocytes disaggregated from the complex could not incorporate these labelled substances, although they could

incorporate labelled amino acids. In regenerating liver, after partial hepatectomy, at the time of maximum mitosis, hepatocytes show a great reduction in the size and number of gap junctional plaques (Yancey et al, 1979).

A few deficiencies of gap junctions have been described; in man, hereditary mucoepithelial dysplasia, an autosomal dominant trait, produces deficiencies in keratinization and cell adhesion of the epithelia of all mucosal orifices, is thought to be due to a reduced ability to form desmosomes, hemidesmosomes and gap junctions (Witkop et al, 1978; 1979). Mice with a recessive mutation at the T/t complex locus, homozygous for the t^9 allele are histologically abnormal at 9 days gestation, with an enlarged primitive streak and a deficiency of mesoderm. The mutant mesoderm exhibits very small and sparse gap junctional plaques, while possessing increased amounts of cell apposition in comparison to normal mesoderm and the embryo dies in utero (Spiegelman, 1976). Other mutations such as Splotch and amputated in mice show defects in neural tube closure and skeletal abnormalities respectively. The neuroepithelial cells of Splotch homozygous mice contain increased numbers of gap junctional vesicles, thought to be structures formed during gap junction degradation, prior to the development of visible abnormalities (Wilson and Finta, 1979). The amputated homozygous mice show increased incidence of gap junctions in the sclerotomes, and subsequently develop skeletal abnormalities and die at term (Flint and Ede, 1978). The role played by gap junctions in these mutants is unclear and speculative, but the role of junctions in organisms which are hemizygous for some enzyme mutations is more fully documented.

As most tissues have gap junctions, except skeletal muscle, some nerve cells and circulating blood cells, it follows that any

organism which is hemizygous for a particular X-linked mutant metabolic enzyme, will exhibit a metabolic phenotype which is much closer to the wild type phenotype than the mutant phenotype if the deficient metabolite is small enough to pass through the junctional channel. This phenomenon has been demonstrated in hemizygous females for the X-linked trait responsible for Lesch-Nyhan syndrome, a neurological disorder associated with the absence of HGPRT activity in homozygous sufferers. In hemizygous females, due to random X-chromosome inactivation, there exists two populations of cells, one $HGPRT^+$ and the other $HGPRT^-$, but the sufferers appear normal (Migeon et al, 1968). Work previously described (see Section 1:4) has demonstrated that in $HGPRT^+/HGPRT^-$ mixed cultures, $HGPRT^-$ cells in contact, directly or indirectly through other $HGPRT^-$ cells, with $HGPRT^+$ cells can incorporate [^{3}H]hypoxanthine (Subak-Sharpe et al, 1966; Subak-Sharpe et al, 1969) and this was shown to be due to the exchange of nucleotides, from wild-type to mutant cells (Cox et al, 1970; Pitts, 1971). Furthermore, it has been demonstrated that 1:1 mixtures of $HGPRT^-$ and TK^- mutant cells grow in the presence of HAT medium, which is toxic to both $HGPRT^-$ and TK^- cells alone, by gap junctional communication (Pitts, 1971). Even in 1:20 mixtures, after initial cell death, the remaining cells grow to form a surviving population, which is self-stabilising, at a 1:1 mixture. This sort of tissue phenotype (a property of cell mixtures not seen in isolated cell types) is also seen in co-cultures of human wild type and rodent TK^- cells in the presence of [^{3}H]-thymidine and ouabain at concentrations which inhibit the primate type Na^+/K^+ ATPase, but not the rodent enzyme. Incorporation of the label is dependent on both cell types being able to form gap junctions (Pitts and Shaw, 1980). The establishment of tissue phenotypes has also been

demonstrated using [^{14}C]-proline as the label in wild type/mutant co-cultures but not in mutant/mutant co-cultures, or wild type/mutant co-cultures which do not form gap junctions (Finbow and Pitts, 1981). However, a more subtle example of tissue phenotype development was demonstrated when wild type and HGPRT$^-$ junction forming cells were grown in the presence of high concentrations of exogenous hypoxanthine. Under these conditions the exogenous hypoxanthine stimulates the HGPRT pathway in the wild type cells and as a consequence inhibits de novo purine nucleotide biosynthesis in both cell types. The incorporation of [^{3}H]-hypoxanthine in 1:10 mixture (wild type to mutant) was threefold higher (on a per wild type cell basis) than in wild type cell cultures growing alone (Sheridan et al, 1979). The probable reason for the increase in [^{3}H]-hypoxanthine incorporation is that mutant cells are taking away, by gap junctional transfer and incorporation [^{3}H]IMP, the product of HGPRT action on hypoxanthine, synthesised in the wild type cells.

From the above in vitro examples, it must follow that cells with metabolic defects may accumulate large amounts of the substrate of a defective enzyme and lack the product. If the substrate and enzyme product are small enough to pass through gap junctional channels, and if the mutant cell is coupled to wild type cells, then concentration gradients will be established so the substrate will flow from the mutant to wild type cells, where functional enzyme will act as a sink for the substrate, and the product will pass down the concentration gradient from the wild type to the mutant cells as the product is not made by the mutant.

Tumour cells have been shown to lack or have impaired junctional communication (Loewenstein, 1979) and as such could be alienated or excluded from the normal homeostatic influences of surrounding

normal cells. It may be possible that reduction or loss of gap junction mediated comunications is causal or contributory to the abnormal phenotype that tumour cells express. The exact role of junctional communication in development, tissue induction and differentiation has yet not been elucidated, but the loss or reduction of gap junctional communication may increase the probability that an aberrant cell will become frankly abnormal or neoplastic.

Thus, junctional communications in animals would appear to be involved in development, tissue induction and cell and tissue differentiation, providing co-ordination, synchrony, stability in excitable tissues and metabolite exchange and co-operation in tissue function. However, it is unknown what profound consequences alterations in gap-junction formation and organisation may have on the organism in health and disease.

1:6 Cellular interactions in haemopoiesis

In the normal adult one twentieth of the total blood cell population is lost daily, requiring the generation of new cells to make up for those removed. The source of these new cells is the bone marrow which generates five different types of cells, lymphocytes, monocytes, granulocytes, erythrocytes and megakaryocytes. The bone marrow consists of stromal cells arranged into vascular networks set in a framework of multi-branched reticular cells within which haemopoiesis progresses (Weiss, 1970; Campbell, 1972; Shaklai and Tavassoli, 1979). The stromal cells provide a microenvironment in which haemopoietic stem cells proliferate and differentiate (Trentin, 1971; Tavassoli, 1977; Ploemacher, 1978).

The importance of this microenvironment is demonstrated in the Sl/Sl^d mouse, which has normal haemopoietic stem cells as it can

restore normal haemopoietic function to lethally irradiated mice of the same haplotype but is itself congenitally anaemic (Russell, 1970; Altus et al, 1971; Tavassoli et al, 1973; Shaklai and Tavassoli, 1978). Similar microenvironmental defects have been suggested as the reason for the inability to restore haemopoietic function in identical twins after bone marrow transplantations (Fernbach and Trentin, 1962).

The primary site of haemopoiesis has been identified as the erythroblastic islands of the yolk sac (Sorrenson, 1961), the foetal liver (Zamboni, 1965; Fudaka , 1974; Chui and Russell, 1974) and the spleen (Orlic et al, 1965; Djaldetti et al, 1972). In the bone marrow, erythroid precursors form close associations with non-haemopoietic cell types (Bessis, 1958) where a central macrophage-like cell is surrounded by erythroblast precursors in various stages of differentiation (Marmont and Damasio, 1962).

However, attempts to define interactions between pluripotent stem cells and stromal elements by morphology has been hindered by the fact that as the closer the differentiation stage approaches to that of an immature phenotype, so the morphology of mononuclear cells converge (Yoffey, 1962; Cudkowitz et al, 1964). Estimates have been made of the percentage of stem cells in the bone marrow by reconstituting lethally irradiated mice with varying amounts of bone marrow cells and counting the number of haemopoietic colonies in the spleen. However within the stem cell population which can repopulate lethally irradiated mice, there appear to be differences in proliferative capacity. Brain antigen negative stem cells show a three-fold greater capacity for renewal than their antigen positive counterparts (Monette and Stockel, 1981). Attempts to overcome this heterogeneity by reconstitution and serial transplantation have

suggested that in vivo reconstitution does not affect the potential for self-renewal and that there is no intrinsic limit on stem cell capacity for repeated regeneration. In vivo reconstitution experiments have elicited little concerning the interactions of stem cells and stromal components as the stromal elements are radioresistant, but in vitro assays have demonstrated the requirement of stromal cells and the close interaction necessary for differentiation of stem cell progeny (Lord, 1981). In vitro culture of stem cells without stromal elements produces a very limited range of cell types, primarily erythroid, megakaryocyte and a few granulocyte/macrophage colonies. Whereas in vitro culture of bone marrow on established marrow stromal cultures will produce full differentiation of most cell types with the exception of lymphoid cells and allows the maintenance of stem cells themselves (Dexter and Lathja, 1974). Marrow cells, thus cultured, have been shown to be able to reconstitute the lymphoid component as well as providing precursors for all other cell types in lethally irradiated mice (Schrader and Schrader, 1978; Jones-Villeneuve and Phillips, 1980). However, only recently has a modified Dexter culture method been described which will support the growth and differentiation of lymphocyte precursors, specifically B lymphocytes, which constitute the humoral arm of the immune system (Whitlock and Witte, 1982). In contrast, there have been no reports of the in vitro cultivation of the lymphoid component of the cell-mediated arm of the immune system, the T lymphocytes.

Histological analysis of bone marrow has shown interactions between lymphoid cells and stromal elements (Lord , 1979) and freeze fracture analysis has demonstrated intramembranous particles between haemopoietic and stromal elements similar to small gap junctional plaques, as well as close membrane apposition (Campbell,

1980). Freeze fracture and thin section electron microscopy of *in vitro* cultures of canine bone marrow and rat bone marrow *in vivo* have demonstrated gap junction-like structures in monocyte/macrophage colonies but not in granulocyte/macrophage colonies (Porvasnik and MacVittie, 1979; Tavassoli and Shaklai, 1979). In 1976 Levy reported electrical coupling between peritoneal macrophages (Levy *et al*, 1976), but subsequent work has failed to demonstrate metabolic coupling (Kane and Bols, 1980). This disparity is most probably due to the greater sensitivity of electrophysiological methods in comparison with metabolite exchange methods.

1:7 The Immune System

The earliest self recognition system appears in *Porifera* where, if the cells of two sponges are mixed they will segregate into the original discrete entities, but it is not until the *Coelenterates*, *Tunicates* and primitive vertibrates, that a primitive cell-mediated immunity and antigenic memory appears. Humoral immunity, however, does not appear until the primitive fishes. Every organism from cyclostomes upwards, exhibit circulating lymphocytes, but it is not until the hagfish, that lymphoid cells in the gill arches form any lymphoid tissues. Bony fish possess a thymus, but at this point in evolutionary development there seems to be no secondary lymphoid tissue organisation.

In higher animals, two arms of the immune system have been well defined, the cell mediated arm and the humoral. The cell mediated arm is phylogenetically older than the humoral arm. The humoral arm consists of lymphocytes which bear and secrete antibody molecules in response to antigenic challenge. The generation of these specialised lymphoid cells is best seen in *AVES*. In birds stem cells

migrate from the bone marrow to the Bursa of Fabricius, an organ of specialised epithelium off the cloaca, where they mature to surface immunoglobulin positive cells (Cooper et al, 1966). On bursal maturation, these bursal lymphocytes, or B cells, migrate to secondary lymphoid organs and populate the thymus independent areas of the peripheral lymphoid tissues (Hemmingian and Linna, 1972).

The production of antibody producing cells is dependent on the bursa and the interactions of migrant lymphoid cells with the stroma cells of the organ (Schaffner et al, 1974). Neonatal bursectomy, lethal irradiation and bone marrow reconstitution produces birds which have intact cell mediated lymphoid function, but which are agammaglobulinaemic. Bursal lymphocytes from antigen primed bird are capable of producing secondary antibody responses in unprimed fowl recipients after transfer and antigenic challenge (Gilmour et al, 1970).

The bursal equivalent has not been found in mammals although many workers have attempted to identify the tissue in which B lymphocytes differentiate in association with stromal elements. It was initially thought that the gut associated lymphoid tissues (GALT) were the mammalian bursal homologue (Archer et al, 1963; Sutherland et al, 1964; Cooper and Lawson, 1974; Nieuwenhuis et al, 1974). However, it is now generally accepted that foetal liver (Owen et al, 1974; Sherwin and Rowlands, 1974; Phillips and Melchers, 1976) and subsequently the bone marrow, show first signs of immunoglobulin producing cells and function as the mammalian bursal homologue (Abdou and Abdou, 1972; Lafleur et al, 1973; Brahim and Osmond, 1973; Phillips and Miller, 1974). As antibody producing cells leave the bone marrow in a state little different from that of responsive circulating B

cells in the periphery it must be assumed that the stromal interactions, which allow them to become committed to the B cell lineage and differentiate, occur in the bone marrow.

The cells of the cell-mediated arm of the immune system also arise by differentiation from pluripotent stem cells in the bone marrow. In the marrow these cells are committed to the cell-mediated lineage but are undifferentiatied at the level of responsiveness to the antigenic environment (Zinkernagel, 1978) with the possible exception of allospecific non-primed cytotoxic cells observed in the yolk sac and foetal liver (Triglia et al, 1981). The committed unreactive cells leave the bone marrow, pass through the periphery and are sequestered by the thymus, hence the name T lymphocytes (Ford and Micklem, 1963; Moore and Owen, 1967; Jotereau et al, 1980). This interaction between committed cells and the thymus is an absolute requirement for the differentiation of T cells (Miller and Osoba, 1967; Greaves and Janossy, 1973; Cantor and Weissman, 1976; Stutman, 1978). A feature of some lymphoid cells in the bone marrow is the presence of the nuclear enzyme, terminal deoxynucleotidyl transferase (TdT; EC 2.7.7.31) (Coleman et al, 1974; Paz mino et al, 1977) a DNA polymerase, which does not utilise a template. It has been proposed that its presence provides a means of generating diversity, of immunologic repertoire in prothymocytes (Baltimore , 1974) and in the rearrangement of heavy and light immunoglobulin genes in B cell precursors from germ line genes (Early et al, 1980).

1:8 T Lymphocytes

To describe T lymphocyte differentiation in the thymus, it is necessary to describe the characteristics of functional T cells in the periphery. T cells by definition require functional thymus to

elaborate their activities and functions. The functional heterogeneity of T lymphocytes is well established (For review see Cantor and Weissman, 1976) and antisera are available which define cell surface antigens. One group of antigens, the lymphocyte thymus antigens (Lyt) 1,2,3,6,9 and 10, (Cantor and Boyse, 1975a; Cantor and Boyse, 1975b; Cantor and Boyse, 1977) which are co-expressed on the majority of thymic lymphocytes, are differentially expressed on functionally distinct subsets of peripheral T lymphocytes. Initially three major subsets of peripheral T cells were described: Lyt$1^+2^-3^-$, Lyt $1^+2^+3^+$ and Lyt $1^-2^+3^+$. Subsequent analysis has shown that the Lyt 3 antigen is located on the same macromolecule as the Lyt 2 antigen (Ledbetter and Herzenberg, 1979) and high titre monoclonal antibodies have revealed that all T cells carry the Lyt 1 antigen. Two populations have since been defined, Lyt 1^+2^- and Lyt 1^+2^+, with variation in the amount of Lyt 1 antigen in the Lyt 1^+2^+ subset. Functional T cells divide into four groups: antigen induced cytotoxic cells (T_C) and suppressor cells (T_{supp}) which bear the Lyt 1^+2^+ phenotype and helper cells (T_H) and delayed type hypersensitivity (T_{DTH}) cells which bear the Lyt 1^+2^- surface phenotype (Rock et al, 1983).

A unique property of T_C and T_{DTH} cells is that the 'foreign' antigen receptor expresses specificity for determinants encoded for by the major histocompatibility complex (MHC) locus. This phenomenon has been called MHC or H-2 and HLA restriction in mouse and man respectively and was first observed in experiments examining the T_C killing of virally infected targets by virus primed lymhocytes (Zinkernagel, 1976). Briefly, it was observed that T_C from virus primed mice would only killed virus infected targets if both the T_C and target shared the same MHC haplotype. Primed T_C would not kill virally infected target cells which differed from the T_C at

the K or D loci of the MHC, but would kill infected targets from different mouse strains which shared the same K and D haplotype. This phenomenon was first described for products of the K and D region of the mouse MHC (Zinkernagel et al, 1978a; 1978b; Zinkernagel and Doherty, 1979) but examination of the interactions of effector cells of the immune response and T_H cells, revealed that T_H cells possess receptors for proteins encoded for the I region which lies between K and D regions of the MHC (Erb and Feldmann, 1975; Kappler and Marrack, 1976; Lutz , 1981). Further examination has revealed that I region restriction is at the level of the interaction of T_H cell with an accessory or antigen presenting cell, not at the effector cell level (Vogt et al, 1981) and that I region restriction applies to Lyt-1^+2^- T_{DTH} cells (Miller et al, 1975).

Although Lyt 1^+2^+ T_C precursors exhibit H-2K/D restriction, they can also generate specific receptors against allogeneic H-2K/D, probably H-2I and other MHC linked surface determinants such as Qed-1 (Bevan, 1975; Peck et al, 1976; Hansen and Levy, 1978).

Interest in these receptors was intensified by reports that the MHC haplotype of an engrafted thymus determined the reactivity of T_C cells and H-2 restriction (Fink and Bevan, 1978; Zinkernagel, 1978; Zinkernagel et al, 1980) to the haplotype of the engrafted thymus not the haplotype of the host. Considerable controversy has raged as to whether the surface proteins that detect self and 'foreign' antigens are two separate receptors or a single molecule which can bind both H-2 and 'foreign' antigens, but recent evidence points to dual specificity rather than two separate receptors (Hunig and Bevan, 1980; Kappler et al, 1981).

The T cell receptor, unlike the B cell receptor, the immunoglobulin molecule, has been a very difficult molecule to define.

Initial attempts to analyse this surface molecule were focused on cloned T cell tumour lines (Allison et al, 1982) and later on hybrids of T cell tumour lines and T cell lines of known antigen and/or MHC specificity (Kappler et al, 1981) which lacked independent antigen and MHC recognition. Xenoantiserum raised against tumour cells recognised molecules with structural heterogeneity on normal T cells (McIntyre and Allison, 1983) and monoclonal antibody raised against T cell hybridomas recognised MHC restricted antigen receptors (Haskins et al, 1983), while anti-idiotypic antibodies predicted both antigen and Class II MHC specificity (Marrack et al, 1983).

The protein isolated was biochemically characterised as a disulphide linked heterodimer with subunits of aproximately Mr 45k (Allison et al, 1982; Meuer et al, 1983(a); Haskins et al, 1983). Subsequent investigations have shown the dimer to consist of an α subunit of Mr 49-51k and a β subunit of Mr 43k(Acuto et al, 1983a). Subsequent biochemical characterisation by two-dimensional peptide mapping identified constant and variable peptides (Kappler et al, 1983) which show variability in the variable and constant peptides in α and β subunits from T cell clones of different specificities (Acuto et al, 1983b). The β chain has been purified and the NH_2-terminal amino acid sequence has been ascertained (Acuto et al, 1984).

Analysis of T cell receptor proteins of IL-2 dependent T cell clones with antibodies directed against idiotypic determinants (Ti molecules) and antibodies directed against the T cell surface protein T3, a three subunit molecular complex found on peripheral T cells and thymocytes with a mature phenotype, have shown an association on the cell surface of T cell receptor (Ti) and the Mr 20K unglycosylated and glycosylated 20 and 25k T3 complex (Meuer et al, 1983b). This finding has been extended to show that the Ti/T3

membrane complex is not expressed on thymocytes until genes encoding the Tiβ sub-unit undergo rearrangement (Royer et al, 1984).

Genes for the β chain of the heterodimer were recently isolated (Yanagi et al, 1984; Hendrick et al, 1984) and showed extensive homology to the immunoglobulin chains. Analysis of these genomic sequences has indicated that diversity of the β chain is generated to some extent by somatic rearrangement of germ line gene sequences (Chien et al, 1984a; Gascoigne et al, 1984; Malissen et al, 1984) and have been localised to chromosome 6 in mice and 7 in man (Caccia et al, 1984). Later, Saito et al (1984b) and Chien et al (1984b) isolated genes which probably code for the α chain protein of the heterodimer and also undergo rearrangement from their germ line configuration as in the β chain genes, to generate diversity. An article by Saito et al, which appeared in the same issue of Nature as the Chien paper (Chien et al, 1984a), described a cDNA clone which was thought to encode the α chain product (Saito et al, 1984a). Although the genes showed similarities to both Ig and β Ti genes in organisation and sequence, they were expressed only in T cells. They, or genes highly homologous to them, were expressed in all cytotoxic T cells examined, but do not encode either of the α or β genes previously described and have been called the γ genes.

The arrangement of the gene segments of α, β and γ Ti genes on the chromosome is similar to that of the light and heavy chain gene segments of the immunoglobulin genes, i.e Variable (V) - Diversity (D) - Joining (J) - Constant (C), but they exhibit arrangements which are in some ways like light chain segments and others which are like heavy chain segments. In the β and α loci there is an unknown number

of V segments, but only three V region segments have been identified in the γ loci. The β genes exhibit two D segments unlike κ and λ light chains in a tandem arrangement D_1-J-C-D_2-J-C, but it is not known if α and γ loci contain D elements.

The J segments of the α loci have not been described but preliminary data suggest that they display combinational and junctional diversity (Saito et al, 1984b; Chien et al, 1984b). The γ loci appears to exhibit three J segments which are arranged in a similar fashion to the λ light chain arrangement, i.e J_1-C_1, J_2-C_2, J_3-C_3. The β J region consists of seven J elements of the $J\beta_{1.1}$ separated from seven more J elements by the $C\beta_1$ element. This resembles the five J elements of the κ light chain and the four heavy variable chain regions of Ig, in that the J elements are grouped together before the C region elements. However, it also resembles the λ light chain elements where each of the four J segments is separated by an intervening C element (Hood et al, 1985).

The first exon on the C region genes encodes a 110 amino acid domain by its length and conservation of structurally significant residues it resembles an Ig C domain. While V-J segments also resemble Ig domains the coding capacity of J segments of Ig κ and λ is very similar to that of α, β and γ J segments, but the γ J segments are more similar to β J segments than Ig J segments. The V and J segments are flanked but heptamer and nonamer sequences which are identical in organisation and similar in sequence to Ig. All three Ti coding genes appear to undergo junctional diversity from the germ line configuration but the $C\gamma$ genes do not possess a great capacity for combinatorial diversity, as one of the $C\gamma$ segments appears to be defective, unlike the α and β genes. The other major source of

diversity seen in Ig is hypervariability caused by somatic mutation of rearranged V regions, but has not been observed in the few cases thus far studied. However, this response is predominantly seen in IgG rather than IgM and is considered to be an outcome of mature responses but it has been observed that T cell hybrids frequently lose the ability to respond to expected stimuli, but in a recent study typtic peptide maps of such hybrids show different patterns in both α and β chain gene products (Augustin and Sim, 1984).

Thus, the discovery and investigation of the T cell receptor at the genomic and protein level has yielded information as to the structure and constituent parts of the T cell receptor the problem of what the receptor 'sees' in MHC restriction has not been resolved (Zinkernagel and Doherty, 1979; Matzinger, 1981; Hunig and Bevan, 1982). The presence of three groups of genes found in T cells does not rule out the possibility that three types of heterotypic receptor dimers could be produced, i.e $\alpha\beta$, $\alpha\gamma$, $\beta\gamma$, nor is there any information about the possible formation of homotypic receptor dimers. Further work on the ontogeny and regulation at genomic, RNA and protein levels may give insight into the phenomena of T cell recognition.

However, recent reports suggest that anti-self H-2 receptors may be expressed prior to entry into the thymus and haplotype specificity difference in the spleen in T_C cells and their precursors (Kruisbeek et al, 1981; Morrissey et al, 1982) have complicated the initial observations of Zinkernagel et al, (1976). Induction of T_C cell function requires in addition to T_C cell precursors, Ia positive antigen presenting cells (Farrar et al, 1980), T_H cells (Finberg et al, 1979) and a factor which induces T_C cell differentiation (Raulet and Bevan, 1982).

The correlation of Lyt surface phenotype with functional phenotype as recently been placed in doubt by a report that Lyt 2^+ cells are apable of 'helping' B cells if there exists a difference in the H-2K or D region of the MHC between the B cells and T_H cells (Swain and Panfili, 1979). Furthermore, a Lyt 2^- cell line has been developed which is specifically cytotoxic for products of the H-2I region (Dennert et al, 1981; Swain et al, 1981). A hypothesis has been proposed that Lyt antigens may mark T_C cells which are either H-2K/D region or H-2I region specific to account for the seeming disparity between surface Lyt antigen phenotype and function (Swain et al, 1981). This hypothesis contrasts strongly with presented data that in vitro generated primary anti H-2I region T_C cells bear Lyt-2 as do anti H-2K/D region specific T_C cells (Miller and Stutman, 1982; Rock et al, 1983).

The other major subset of mature T cells is the Lyt 1^+2^+ suppressor cells (T_{supp}), which regulate the activity of B cells, T_C cells and T_{DTH} cells (Taniguchi et al, 1976; Kontiainen and Feldmann, 1977; Lieu, 1981). T_{supp} cells are thought to produce a product(s) which act on Lyt 1^+2^+ cells as amplifiers of suppression rather than effectors (Germain and Benacerraf, 1980). This factor(s) secreted from T_{supp} has direct suppressive action on in vitro B cell antibody production and T_C function and produces specific antibody responses in H-2I region incompatible recombinant inbred mice (Lieu, 1981; Yamauchi et al, 1981). Furthermore it has been reported that cell-free translation products of poly A-mRNA from an AKR lymphoma/splenic suppressor cell hybrid produces an antigen specific biologically active factor (Weider et al, 1982).

Recently a series of alloantigens has been described on T_H and T_{supp} cells which do not bind antigens, but are expressed on Lyt

1^+2^- and Lyt 1^+2^+ cells called $Tind^d$ and Tsu^d respectively. Injection of antisera against Tsu^d into mice causes suppression of B cell responses, whereas injection of antisera against $Tind^d$ causes cyclic waves of suppression and enhancement of B cell responses. These alloantigens are encoded by genes downstream of the immunoglobulin locus on mouse chromosome 12 and are linked to the Igh-1 genes (Spurll and Owen, 1981). Using an anti-idiotypic antibody raised against an antibody which binds H-2I region determinants, a receptor which binds self Ia antigens has been identified and its expression is controlled by genes which map close to the Igh-1 locus (Nagy et al, 1982).

As better probes for surface antigens are evolved, the heterogeneity of surface antigen phenotypes of T cell subsets and their correlation with functional phenotypes may be resolved (Hiramatsu et al, 1982; Hayes and Hullet, 1982; Fukumoto et al, 1982). Above is presented a simplified overview, which attempts to explain and elucidate the complex interactions of functional T cell subsets in relation to their surface antigens as they are currently understood. However, if idiotypic and allotypic regulation are also considered, the network of possible interactions becomes exceedingly complex.

1:9 The Thymus

The thymus is a white to grey gelatinous encapsulated bilobed organ which lies posterior to the clavicle and superior to the heart. It is supplied with blood from the first arch of the aorta but possesses no afferent lymphatic ducts, only efferent ducts which drain to the parathymic lymph nodes on either side of the lobes of the organ (Weiss, 1980). The organ is derived from the interaction of the embryonic ectoderm and endoderm of the third pharyngeal cleft

and pouch respectively without intervening mesenchyme (Chiscon and Golub, 1972; Cordier and Heremans, 1975). The epithelial and endothelial cells, separated by a basement membrane, are set in a matrix of extracellular material and cells of neural crest origin derived from the pharyngeal arch mesenchyme (Jenkinson et al, 1981).

In perinatal and neonatal life the thymus is a comparatively large organ which decreases in size after puberty as the lymphoid cell content decreases. As the lymphoid content decreases, there is concommitant increase in 'adipocyte'-like cells and in adulthood the organ becomes involuted. Premature involution can be induced by the injection of corticosteroids (Ezine and Papiernik, 1981) and pregnancy causes transient involution (Phuc et al, 1981). Only in certain immunopathologies is the trend to decrease in organ size not seen (Lampkin and Potter, 1958).

The thymus is architecturally divided into cortex and medulla. The cortex is a layer many cells in thickness characterised by the branching of arterioles into capillaries at the cortico-medullary boundary. The outer cortex consists largely of medium to large lymphoblasts with the mid cortex and juxta-medullary cortex containing small densely packed lymphocytes. Throughout the cortex the capillaries possess tight junctions and are surrounded by macrophages. At the cortico-medullary boundary the capillaries form post-capillary venules with characteristic high sided endothelial cells. In contrast the medulla contains much lower concentrations of lymphocytes, less densely packed with a predominance of small to medium cells (Cantor and Weissman, 1976).

The differentiation of the thymus into cortex and medulla can be defined by immunological criteria. The cortex contains a mixture of cells, functionally immature, with or without newly acquired mature

surface antigen phenotypes. The medulla contains functionally mature cells with a mixture of immature and mature surface antigen phenotypes. Clark (1968) demonstrated that mitotically active cortical cells gave rise to the small inactive lymphocytes of mid- and juxta-medullary cortex with a proportion of medullary cells undergoing mitosis (Clark, 1968).

1:10 Intrathymic Lymphocyte Development

Considerable effort has been expended on discriminating precursors, intermediate stages and maturing T cells in the thymus by examining function, cellular enzymes and surface antigens (for review, see Cantor and Weissman, 1976). Historically, initial investigations relied on techniques which removed or ablated thymus function. Thymectomy in early neonatal life showed that both cell mediated responses and T cell dependent B cell responses were lost in later life, but thymectomy after 6 weeks in mice and 6 months in man impairs neither function, although a reduction in T_H cell memory was observed (Metcalf, 1960; Miller, 1965). The other technique utilized was the injection of cortisone, which causes lysis of ≈ 90% of thymic lymphocytes (Weissman, 1973). Initially, cortisone treatment was thought to affect only cortical thymocytes, without any apparent alteration in medullary thymocytes, but subsequent work has suggested that medullary thymocytes are also affected by in vivo cortisone treatment (Irlé et al, 1979). However, it should be noted that cortisone sensitivity of thymocytes varies from strain to strain in mice (Roelants et al, 1979). The residual thymocytes, after in vivo cortisone treatment, exhibit some of the characteristics of peripheral T cells; i.e PHA and CON-A responsiveness, MLR responsiveness, ability to induce Graft versus Host (GVH) disease,

production of cytotoxic lymphocytes (CTL) on stimulation, low $Thy1^+$ surface antigens (ag), high H-2K/D ag, TL^- ag, and low numbers of $Qa\text{-}2^+$ cells. Subsequent experimental observations have shown that some 'immature' phenotype cells are resistant to cortisone while some 'mature' cells are cortisone sensitive and that 'mature' thymocytes in vitro are cortisone sensitive (Triglia and Rothenberg, 1981). Recent observations on cortisone sensitivity has given support to the suggestion that cortisone resistance may be conferred on thymocytes by a thymic humoral factor(s) or cell-cell interaction in the thymus (Trainin et al, 1974).

From the observations of action of cortisone on thymic lymphocytes and microdissection of the thymus into cortex and medulla, the theory evolved that prothymocytes were sequestered to the outer cortical region, underwent differentiation and as differentiation proceeded, thymocytes migrated to the medulla. This theory has persisted until recent experiments using quail thymic grafts to chickens and vice versa in ovo, where it was observed that the thymic homing precursor lymphocytes migrated to both the cortex and medulla (Jotereau and Le Douarin, 1982).

Cellular enzymes in the thymus have been studied in attempts to separate subsets of cells. Among the enzymes studied have been DNA ligase, TdT and sialyl transferase. In studies on DNA ligase in calves, different types of enzyme in thymocytes were observed: a large 8.2S type and a smaller 6.2S type (Söderhall and Lindahl, 1975). In more recent studies in chickens, the large 8.2S type ligase is associated with large surface antigen negative thymocytes, whereas large surface antigen positive cells were devoid of any ligase activity, but small antigen positive thymocytes contain the 6.2S form of the enzyme. The 8.2S form has been associated with DNA

replication and the 6.2S DNA ligase with DNA repair (David et al, 1981). Other enzymes which have been studied in thymocytes include TdT, adenosine deaminase (ADA) (EC 3.5.4.4), purine nucleoside phosphorylase (PNP) (EC 2.4.2.1) and 5' nucleotidase (EC 3.1.3.5).

TdT, as previously mentioned, catalyses the addition of deoxynucleoside triphosphates to the 3'OH end of DNA without a template. This enzyme is restricted to the bone marrow and thymus and although it has been purified and biochemically characterised, its physiological function is unknown (Modak, 1979). It has been suggested that it functions as a somatic mutagen (Baltimore, 1974) and could contribute to the generation of diversity in the immunologic repertoire (Bollum, 1978). Initial interest in TdT centred on its occurrence in bone marrow and thymus, its age and cell maturation associated decline (Pahwa et al, 1981), its possible role as a marker for T lineage commitment in marrow (Pazmino et al, 1977) and prothymocytes in the thymus (Silverstone et al, 1976). TdT^+ cells isolated from bone marrow have been shown to be early B cells (Whitlock and Witte, 1982) or null cells (Schrader et al, 1979) but TdT has been demonstrated in mouse and human thymocytes. Although in foetal and neonatal mice the activity of the enzyme is low, it increases to adult levels one week after birth. In adult mice, large proliferating thymocytes do synthesise TdT, but small non-dividing cortical cells devote equal or greater proportions of total protein synthesis to the production of TdT. In late foetal and neonatal mice, although TdT production is low, overnight in vitro culture induces TdT production. Treatment with a monoclonal antibody against the intrathymic and peripheral T cell surface antigen, Lyt 2, and complement lyses the population of thymocytes which change from TdT^- to TdT^+ after in vitro culture

(Rothenberg and Triglia, 1983). TdT production is induced in PHA stimulated normal human peripheral blood lymphocytes during in vitro culture, but given the bone marrow and thymus restriction of TdT^+, the identity of these TdT^+ cells is unknown unless they are circulating B and T cell precursors.

Interest in TdT, ADA, PNP and 5'NT was initially stimulated by the observation that individuals homozygous for ADA and PNP mutations suffer from Severe Combined Immunodeficiency (SCID). These mutations are very rare and give rise to clinical presentations of infants which fail to thrive and are prone to infection. On assessment of immunological function they show little or no DTH or PHA and CON-A responses, rarely make functional antibody, exhibit reduced or absent thymic shadow and have hypotrophic or involuted thymuses on autopsy (Giblett et al, 1972; Giblett et al, 1975; Parkman et al, 1975). ADA and PNP act sequentially in the purine salvage pathway, with ADA irreversibly catalyzing the conversion of adenosine to inosine and PNP reversibly catalyzing the conversion of inosine or guanosine to hypoxanthine and guanine respectively.

Inhibition of ADA, by the inhibitor coformycin, inhibits the production of T cells from human precursor lymphocytes (Ballet et al, 1976). In vitro studies have shown that both coformycin and excess 2'-deoxyadenosine (dAr) inhibit the maturation of cortical thymocytes and inhibit immunocompetent medullary thymocyte responses to Con-A stimulation (Thuillier et al, 1981). In studies examining the distribution of ADA in rat and human thymuses, it was found that cortical cells exhibited higher concentrations of ADA than medullary thymocytes (Barton et al, 1979; Chechik et al, 1981). Similarly, there was found to be an inverse relationship between ADA and PNP concentrations in thymocytes and peripheral T cells, with cortical

thymocytes exhibiting higher levels of ADA and lower levels of PNP than medullary cells and medullary cells showing higher levels of ADA and lower levels of PNP than peripheral T cells (Barton et al, 1980).

It has also been demonstrated that deoxyguanosine (dGr) exposure causes intracellular dGTP accumulation and cell death in thymocytes and the inhibition of peripheral T cell growth even when PHA or CON-A stimulated (Cohen et al, 1980; Kefford and Fox, 1982). The toxic effects of either ADA inhibition or dAr substrate excess have been shown to inhibit T cell growth factor (TCGF or IL-2) production in immunocompetent T cells (Thuillier et al, 1981) and dGr has been employed to clear embryonic thymus rudiments of the lymphoid components (Jenkinson et al, 1982). dGr was also used to inhibit the production of cytotoxic/suppressor cell precursors (Dosch et al, 1980) and a series of human pro-thymocyte lines were protected from the toxic effects of dGr by prior treatment with the tumour promotor 12-tetradecanoylphorbol-12-acetate (TPA). Not only did TPA treated cells gain resistance to dGr, but TdT activity disappeared, ecto5'NT activity increased 3-fold, and surface markers of medullary thymocytes and peripheral T cells were expressed (Sacchi et al, 1983).

5'NT is an enzyme which is primarily located on the outer surface of the lymphocyte cytoplasmic membrane and has the following relative activities with nucleotides: CMP=UMP=AMP>IMP>GMP>dAMP>dCMP>dTMP. The intracellular 5'NT pool is equal to about 10% of the membrane bound form with the majority of dephosphorylating activity showing no specificity for any substrate, suggesting that the majority of this activity is attributable to cytoplasmic phosphatase(s) rather than 5'NT (Conklyn and Silber, 1982). The role that has been suggested for 5'NT is to allow ribonucleotides and deoxyribonucleotides to

be converted to their corresponding nucleosides enabling their transport across the cell membrane as nucleotides will not enter or leave cells unless dephosphorylated (Wortman et al, 1979). It has been reported that thymocytes exhibit no 5'NT activity, but other reports claim that putative prothymocytes contain moderately high 5'NT activity, whereas cortical thymocytes have low 5'NT activity and increased 5'NT is seen in medullary thymocytes and peripheral T cells (Barton et al, 1976). It has been proposed that thymocytes can be defined by their enzyme phenotypes, with large blast cells (putative prothymocytes) containing high levels of TdT, ADA and deoxynucleoside kinase and moderately high levels of PNP and 5'NT. Cortical thymocytes, on the other hand, while being the predominant cell type, contain moderately high levels of TdT and ADA but low concentrations of PNP and 5'NT. Medullary thymocytes, consisting of about 15% of thymocytes have low levels of TdT, ADA and kinases, but high levels of PNP and 5'NT like peripheral T cells (Ma et al, 1982; 1983).

It has been reported that TdT activity is inversely related to sialyl transferase activity. Sialyl transferase catalyzes the addition of sialic acid moieties to the terminal sugar residues of carbohydrate side chains of glycoproteins. Prothymocytes and cortical thymocytes have been shown to have fewer terminal sialic acid residues (J. P. Banga, Personal communication) and lower sialyl transferase activity than medullary thymocytes and peripheral T cells (Despont et al, 1975). The reduction of sialic acid moieties on some thymocyte glycoproteins has been used to separate two populations employing peanut agglutinin (PNA) which binds to β-D-Galactose residues (Reisner et al, 1976), which are blocked by terminal sialation. Increasing concentrations of PNA will agglutinate increasing numbers of thymocytes

up to a maximum of 96%, but at levels conventionally used, approximately 80-90% of all thymocytes will be agglutinated. However, PNA binding in mid-foetal thymuses (2 weeks gestation) is at about a third of this level, but increases to 80-90% between 1 and 4 days after birth (Berrih et al, 1981).

PNA agglutination of thymic lymphocytes has been used to define subsets of thymocytes in conjunction with antibodies directed against thymocyte surface antigens. Historically, the Thy-1 antigen, was observed on peripheral T cells, when cells of one Thy-1 allotype were injected into mice of a different allotype, an antibody was produced which would bind to T cells of the injected allotype and also lymphocytes in the thymus of that Thy-1 allotype. It was found that PNA^+ cells bore high levels of the Thy-1 antigen, were cortisone sensitive and expressed low levels of the MHC locus products H-2K/D on their surface. PNA^- cells expressed the opposite phenotype, but it is not known if the high Thy-1, low H-2K/D PNA^+ cells are the precursors of the low Thy-1, high H2K/D, PNA^- cells as has been suggested (Weissman, 1973; Fathman et al, 1975) or whether they represent separate largely independent lines of thymus lymphocyte development (Shortman and Jackson, 1974; Hopper and Shortman, 1976).

The Thy-1 antigen appears on $Thy\text{-}1^-$ thymus immigrating cells within three hours of their entrance to the organ (Lepault and Weissman, 1981) but recent work using high titre monoclonal antibodies, to examine post-irradiation thymocyte regeneration after bone marrow transplantation, has demonstrated that two different populations of $Thy1^+$ cells develop simultaneously but grow at different rates. High $Thy1^+$ cells and low $Thy\text{-}1^+$ cells, appear and develop simultaneously with both populations containing high proportions of large cycling cells during early regeneration.

According to Kadish and Basch (1976) and Boersma et al (1981), prothymocytes are Thy1$^-$ and in the thymus regeneration experiments the low Thy-1$^+$ population always preceded the high Thy-1$^+$ population. The high Thy-1$^+$ population appears only after a decrease in the relative number of low Thy-1$^+$ cells. All migrating thymocytes were large at the beginning of the repopulation but the first small lymphocytes detected were of the high Thy-1$^+$ class (Boersma et al, 1982). These observations are in conflict with previously reported findings which suggested that the high Thy-1$^+$ phenotype was characteristic of 'immature' immunocompetent cortical thymocytes, but the low Thy-1$^+$ phenotype was indicative of 'mature' immunocompetent medullary thymocytes (Fathman et al, 1975; Cantor et al, 1975; Mathieson et al, 1979). However, they do lend support to the observations of Shortman and Jackson (1974) of independent development of different Thy-1$^+$ subpopulations.

At this point a strong caveat must be introduced with respect to observations on surface antigen phenotype, recent work on the expression of the Thy-1 antigen using monoclonal antibodies has demonstrated that this antigen, which had hitherto been regarded as restricted to a small percentage of bone marrow cells, thymic lymphocytes and peripheral T cells in the haemopoietic system, has been shown to be present on 25-30% of all bone marrow cells. It was found that multipotential stem cells, prothymocytes, some B cell precursors, eosinophils and some immature myeloid cells all bore the Thy-1 antigen and Thy-1 expression could be induced by haemopoietic progenitor cell stimulating factor(s) (Schrader et al, 1982; Basch and Berman, 1982). Not only has Thy-1 expression been observed in cells not of the T cell lineage, it has been demonstrated that isolated Thy-1 molecules from thymocytes exhibit heterogeneity both with respect

to glycosylation and sialation (Hoessli et al, 1980; Carlsson and Stigbrand, 1982). When immunoprecipitated iodinated Thy-1 thymic antigens were subjected to SDS-PAGE there was a marked heterogeneity of apparent molecular weight of the antigens precipitated, indicating molecular weight variability suggesting variable glycosylation. When the same material was separated by isoelectric focusing (IEF), eight different bands were revealed, reflecting variations in sialic acid content (Carlsson and Stigbrand, 1982; Carlsson and Stigbrand, 1983).

If such variation can occur in Thy-1 antigen, probably the most extensively studied thymic cell surface antigen, then perhaps there is similar variability in glycosylation and sialation of other thymic lymphocyte membrane antigens, which may alter the binding of antigenic epitopes by specific antibodies.

Of the many surface antigens present on thymic lymphocytes two antigens, thymus and B cell antigen (ThB) and thymic leukaemia antigen (TL) (in TL^+ mice) are exclusively expressed in the thymus. ThB, although on B lymphocytes, is found on cortical thymocytes but not on medullary cells or peripheral T cells (Eckhardt and Herzenberg, 1980; Scollay and Weissman, 1980). In experiments on the distribution of ThB, cortical and sub-capsular cells were found, by immunofluorescence, to divide into two populations, bright and dull and 50% of thymocytes were positive for this antigen. It was found that in lymphoid cells migrating to the thymus, ThB surface antigen was low or absent, with the percentage ThB^+ cells, 10% at 4 days, 16% at 7 days and 50% at 8 weeks post partum (Haaijman et al, 1981), but very few ThB^+ cells at 4 hours after birth. TL antigen (Boyse and Old, 1969; Rothenberg and Boyse, 1979) is known to be expressed on the surface of 70-80% of all thymocytes in B6-TL^+ mice, specifically

marking cortical cells (Konda et al, 1973; Rothenberg and Boyse, 1979; Scollay et al, 1980). When thymocytes were analysed by indirect immunofluorescence with monoclonal antibody to TL, approximately 80% of the cells were TL^+ with good discrimination between TL^+ and TL^-. Most TL^+ cells were small as defined by light scatter, with 78% TL^+ in the small cell class (82% total) and 82% (0.8% total) TL^+ in the large class, but greater heterogeneity of TL^+ staining in large than in small cells. When cells were fractionated with PNA, TL synthesis of medium and large PNA^+ cells was enhanced, whereas in small cells, compared with unfractionated thymocytes, TL synthesis was reduced sharply, even taking into account their low protein synthesis. There was evidence of TL antigen expression in large PNA^- cells and synthesis was also observed. In vitro culture of unfractionated thymocytes shuts off TL synthesis but synthesis of TdT, H-2 and Lyt2 continues. From these experiments it was concluded that although the majority of thymic lymphocytes bear TL antigen on their surfaces, synthesis is restricted to large thymocyte precursors (Rothenberg, 1982). In both reconstituted irradiated mice and in vitro foetal thymus cultures, it has been found that Thy-1 antigen expression has preceded TL surface expression in immigrant thymocytes (Lepault and Weissman, 1981) and in foetal thymocyte cultures, Thy-1 expression occurs 2-4 days prior to TL surface expression (Kamarck and Gottlieb, 1977).

The expression of Class I MHC products, H-2K and D antigens, has been examined in intra-thymic lymphoid development. Thymus homing cells express high levels of H-2K antigens, in reconstitution experiments, with approximately 75% of the bone marrow derived cells positive at 3-4 hours and approximately 90% positive 24 hours after reconstitution prior to expression of surface Thy-1 (Lepault and

Weissman, 1981). This data is consistant with observations of early thymic stem cells *in situ* (Ritter, 1978) and thymic grafting (Owen and Raff, 1970). High expression of H2K antigens is maintained in outer cortical lymphoblasts in neonatal and adult thymuses and is only diminished in the ontogeny of small cortical lymphocytes (Scollay et al, 1980a). Biosynthesis of H-2K antigens in thymocytes continues throughout intra-thymic development and CMC elimination of Lyt 2^+ cells (approximately 85% of total) in adult mice slightly increases the amount of H-2K antigen synthesis detected (Rothenberg and Triglia, 1981). Increase in H-2K antigen expression was found to preceed TL antigen reduction and loss by 2 days (Abbott et al, 1981) with medullary thymocytes expressing low levels of Thy-1, TL^-, increased H-2K, Lyt 1 and Lyt 2 expression and the acquisition of Qa2,3, as well as responsiveness to CON-A and PHA stimulation (Abbott et al, 1981).

The Lyt antigens, previously mentioned (See Section 1:8), are useful markers for functionally defined T lymphocyte subsets (Cantor and Boyse, 1975a; 1975b; 1977). Analysis of the distribution and epitope density of both Lyt 1 and Lyt 2 has been performed on thymocytes. Monoclonal antibody to Lyt 1 stains 80-90% of all thymic lymphocytes in 3-6 week mice (Mathieson et al, 1979; Scollay and Weissman, 1980), with sub-capsular large thymocytes dividing into Lyt 1^+ and Lyt 1^+2^+ classes. In neonatal thymuses, 14% of all thymocytes stain brightly for Lyt 1 at 4 hours. At 24 hours this proportion has increased to 32% and expanding to 42% at 8 weeks (Haaijman et al, 1981). In PNA^- thymocytes little or no Lyt 2 antigen was synthesised, whereas PNA^+ cells synthesised high levels of Lyt 2 antigen consistant with medullary and cortical thymocytes respectively (Rothenberg, 1982). In experiments by Haaijman et al

(1981), Lyt 1^+ cells were detected both in cortex and medulla, but populations of high Thy-1^+, high Lyt 1^+ and low Thy-1^+, high Lyt 1^+ were also detected. The Lyt 1^+2 and Lyt 1^+2^+ phenotypes seen in all sub-populations of thymocytes, may be independent lines throughout intra-thymic development, residing in the medulla and cortex respectively (Mathieson et al, 1979). This hypothesis has been supported by Scollay and Weissman (1980), but the possibility of a common Lyt 1^+2^+ precursor cell cannot be ruled out.

However, four phenotypically different surface antigen subsets have been described: (1) medium sized, Lyt 2^+, high H-2, low Thy-1, 5% total thymocytes; (2) medium sized, Lyt 2^-, high H-2, low Thy-1, 10% total; (3) small, Lyt 2^+, low H-2, high Thy-1, 60% total; (4) large, Lyt 2^+, low H-2, low Thy-1, 23% total. Populations (1) and (2) are cortisone resistant, and are CTL precursors and IL-2 producers respectively (Ceredig et al, 1982); this data has been supported by another group (Draber and Kieslow, 1981). Although subsets (1) and (2) resemble medullary thymocytes phenotypically the relationship to each other and to the other subsets isolated is as yet unknown.

As it has been calculated that 99% of all thymocytes die in the thymus and the migration rate from the thymus to the periphery is approximately 1% of the total lymphoid cell content per day (Bryant, 1972; Scollay et al, 1980), it is most likely that the major phenotypes experimentally identified mark cells destined to die or are the progenitors of non-functional non-viable cells.

1:11 The Stromal Elements of the Thymus

Differentiation from committed pre-T cell to mature T cell is a complex process involving lymphoid/stromal interactions and humoral

factors. Humoral factor, such as thymocyte stimulating factor (TSF) (Di Sabato et al, 1979), interferon (Lindahl et al, 1974), thymic serum factor (FTS) (Bach et al, 1975) and tumour necrosis factor (TNF) (Abbott et al, 1981) have been shown to affect various stages of intra-thymic lymphoid differentiation.

FTS has been shown to: (1) be produced by thymic epithelium; (2) induce various T cell markers as shown by monoclonal antibodies on immature thymic lymphocytes; (3) induce various T cell functions in immature thymocytes (Dardenne et al, 1982); (4) be absent in thymectomised and 'nude' mice (Dardenne et al, 1980). Bovine thymosin $\beta 4$, a 43 amino acid peptide extracted from the thymus, has been shown in vivo to increase the amount of TdT^+ cells in the thymus of cortisone pre-treated mice by more than 50% (Low et al, 1981). Fraction 5 thymosin and β-1 thymosin have been shown in vitro to treble the amount of TdT^+ cells and induce the expression of Lyt antigens in bone marrow cells and splenic lymphocytes. Three different phenotypes were induced, TdT^+, TdT^+ Lyt^+, TdT^- Lyt^+. In normal mice, treatment induced 80% more TdT^+ Lyt^+ cells from TdT^- Lyt^- bone marrow cells and 80% more TdT^+ cells expressed Lyt antigens. In 'nude' mice, Lyt antigens were induced on TdT^- cells and 80% more TdT^+ cells expressed Lyt antigens after exposure to the peptide (Goldschneider et al, 1981).

It has been shown that thymic epithelial cells possess specific high affinity receptors for oestrogens, androgens and corticosteroids. Treatment of epithelial cells with these different steroids causes the release of factors which have the ability to enhance or decrease lymphoid cell proliferation (Stimson and Crilly, 1981).

Non-lymphoid cells of the thymus contain a component of monocyte/macrophage cells from the periphery whose increase is

concommitant with the acquisition of Ia antigens by the cells of the thymus (Jordan et al, 1979) and increasing age (Jenkinson et al, 1981). Other thymic non-lymphoid cells are: (1) endothelial cells which line the vasculature of the organ; (2) epithelial cells adjacent to and separated from the vascular beds by a basement membrane; (3) stromal mechanocytes (fibroblasts) and reticulum cells in the surrounding mesenchymal matrix. There have been no reports of the function of thymic reticulum cells, although they may be related to or identical to the reticulum cells of the spleen and lymph nodes. The surface antigens of reticulum cells of spleen and lymph nodes have been described in the mouse (Steinman and Nussenzweig, 1980) and are thought to act as stimulators or mediators in primary lymphoid responses (Steinman and Witmer, 1978), syngeneic MLR (Nussenzweig and Steinman, 1980) and produce a soluble factor(s) essential for mitogen responsiveness of T lymphocytes (Klinkert et al, 1980). Reticulum cells share common antigens with many bone marrow cells (Steinman and Nussenzweig, 1980; Beller and Unanue, 1980) and have been placed in the leucocyte lineage (Nussenzweig et al, 1981).

The surface antigens of thymic epithelial cells have been described for normal and 'nude' mice (Jenkinson et al, 1981). 'Nude' (nu^+/nu^+) mice are homozygous for an autosomal mutation, which in pharyngeal derivatives, results in a specific thymic defect (Hair, 1974). While 'nude' mice do possess lymphoid stem cells and committed pre-T cells, normal interactions between thymic stromal cells and lymphoid elements do not occur (Wortis et al, 1971). As a consequence of this failure of interaction, 'nude' mice exhibit a condition which resembles ataxia tangelectasia, Nezelof and Di George syndromes and SCID in man. The result of all these conditions is a total lack of T cell function and a concommitant immunodeficiency

due to the lack of T and B cell interaction. However, CTL have been induced in 'nude' mice in vivo (Wagner et al, 1980) and in vitro (Gillis et al, 1979) by the addition of T_H cell factors from normal mice. In contrast, there have been no reports of helper, suppressor or T cell dependent B cell response induction. It has been demonstrated in 'nude' mice that normal stromal/lymphoid interactions and their consequences (Chiscon and Golub, 1972) do not occur (Cordier and Heremans, 1975) and that thymic cells from normal adults with morphological and ultrastructural characteristics of epithelium bear both H-2K/D and H-2I region products on their surface (Van Ewijk et al, 1980). However, 'epitheloid' cells of the 'nude' mouse thymus bear H-2K/D region products but do not express H-2I region products on their surface (Wekerle et al, 1980; Jenkinson et al, 1981).

Complexes of stromal and lymphoid cells were first described as reticulo-epithelial complexes which could be isolated from intact mouse thymuses by enzyme digestion (Loor, 1979). It was not until these complexes were analysed serologically and ultra-structurally that the epithelial cell component of these structures could be differentiated from 'rosetting' formations found in thymic dissociation protocols (Wekerle et al, 1980). The 'rosetting' structures may be due to the increased adherence of thymic lymphocytes for all stromal cells, non-thymic and thymic, in comparison to peripheral lymphocytes (Luriya, 1979).

The epithelial/lymphoid complexes, also known as Thymic Nurse Cell (TNC) complexes are composed of lymphoid cells resting in sealed membrane invaginations of the epithelial cells which each contain up to 25 small to medium lymphocytes. The nurse cells express H-2K/D and H-2I region products on their surface, but lack expression of Thy-1, Lyt 1, Lyt 2, Qa4, Qa5, surface immunoglobulin and do not

bind PNA. The lymphocytes of the TNC, in the invaginations, appear to be fully intact, metabolically active, display high mitotic activity and are sealed from the external environment as demonstrated by the exclusion of ferritin. The membranes of the nurse cells show tight and gap junctions with adjacent epithelial cells in freeze fracture and exhibit cytoplasmic tonofilament bundles in thin-section electron microscopy, all characteristic of epithelial cells. The membranes lining the invaginations show areas of close contacts with lymphocytes in thin-section electron microscopy and intramembraneous particles resembling gap junctional plaques in freeze fracture electron microscopy. The expression of H-2K/D and H-2I region products by both epithelial cells and macrophages suggested a relationship but this was dismissed due to lack of tight junctions and tonofilament bundles in macrophages and the inability of epithelial cells to phagocytose zymozan particles (Wekerle et al, 1980). These TNCs are found near vascular elements (Hwang et al, 1974) or in the subcapsular area of the thymus (Born and Wekerle, 1982).

1:12 Gap Junction Formation by Peripheral lymphocytes

The evidence for gap junction formation by peripheral lymphocytes is contradictory. It has been reported that peripheral lymphocytes become electrically coupled in the presence of the T cell mitogen phytohaemagglutinin (PHA) (Hülser and Peters, 1972; Oliviera-Castro et al, 1973). PHA causes blast cell transformation in T cells after in vitro culture, but only after 3 days (Unanue et al, 1972; Greaves and Janossy, 1973). This T cell mitogen is thought to function by its ability to bind sugar moieties on surface glycoproteins. As the lectin is tetravalent, it not only binds surface molecules in the plane of the membrane, causing them to be bound together to form

'patches', which the lymphocyte redistributes into a polar 'cap', but also, because of the tetravalent nature of the lectin, lymphocytes agglutinate. The process of 'capping ' and 'patching' is thought to mimic the binding of antigen which stimulates mitosis. However, the electrical coupling data, was gathered during the initial period of lymphocyte agglutination, when there is maximum cell/cell membrane apposition. Resting lymphocytes possess very low membrane potentials of approximately -10mV which rise to approximately -25mV after PHA agglutination (Hülser and Peters, 1972; Oliviera-Castro et al, 1973). Large areas of cell/cell membrane contact is known to give rise to spurious electrical coupling, but freeze fracture replicas of PHA agglutinated lymphocyte membranes display transmembranous particle aggregations resembling gap junctional plaques. However, these particles appear on the endoplasmic (E) face with corresponding indentations on the protoplasmic (P) face (Kapsenberg and Leene, 1979) and, as such, resemble freeze fractured invertebrate junctional plaques (Epstein and Gilula, 1977). Such freeze fracture patterns have never been described in any other vertebrate systems thus far examined. It is unclear whether these structures are true gap junctions, whose freeze fracture morphology has been perturbed by the addition of PHA, or PHA-induced artefacts, or merely artefacts induced by fixation and surface replication (MacIntyre et al, 1974). MacIntyre (1976) reported 'septate-like' junctional complexes on lymphocytes <u>in vitro</u> but little attention has been paid to this observation. It has not been possible to demonstrate metabolic coupling in PHA treated lymphocyte cultures (Cox et al, 1976) in contrast to the reports of electrical coupling and dye transfer. Attempts to demonstrate metabolite exchange between primed CTL and target cells have also been unsuccessful (Sanderson et al, 1977),

as have attempts to demonstrate gap junctions morphologically in the same system (Matter, , 1979), although dye transfer has been reported in the CTL/target system (Sellin et al, 1971) and in PHA treated PBL (Sellin et al, 1971).

There have been no reports of electrical coupling, dye transfer, metabolite exchange, or gap junction structures in untreated lymphocyte cultures. It may well be the case that, the junctions detected electrically in PHA treated lymphocytes, are analogous to the demonstration of electrical coupling in insect epithelial segmental boundaries and late zygote development where electrical coupling, but not dye transfer is detected (See Section 1:5). It is also possible that PHA induced electrical coupling is artefactual, created by large areas of cell/cell membrane apposition or by the induction of transient cytoplasmic bridges brought about by the perturbations of surface molecules and coalescence of individual membranes because of the PHA crosslinking and redistribution of surface molecules. However, PHA induced coupling aside, there have been numerous reports of close membrane interactions involving lymphocytes in the ontogeny and responses of the immune system.

CHAPTER 2

MATERIALS AND METHODS

'Variables won't, constants aren't'

Don Osborn

2:1 Materials

2:1.1 Chemicals

Hydroxy-ethylene piperazine ethano-sulphonic acid (HEPES) and di-methyl sulphoxide (DMSO) were obtained from Koch-Light Ltd; Trypsin (Bovine Pancreas: EC 3.4.21.4), glutamine and heparin from Flow Labs Ltd; May-Grunwald, Giemsa and Rhodamine B stains and Depex were obtained from Gurr & Co. Ltd; Amfix fixer from May and Baker Ltd; AR10 autoradiagraphic stripping film, PAN-F and PAN-atomicX 35mm photographic films, D19 developer and Photo-Flo were obtained from Kodak Ltd; Millipore filters from Millipore (UK) Ltd; glass fibre filters from Whatman Ltd; 13mm diameter glass coverslips from Chance Bros Ltd; Ficoll-400, Ficoll-Isopaque and Percoll were obtained from Pharmacia Ltd; Collagenase Type IV (Clostridium perfringens: EC 3.4.24.3) from Worthington Biochemicals Ltd; Hyaluronidase (Ovine testes, EC 3.2.1.35) injection grade from Fisons Ltd or Worthington Biochemicals Ltd; Dispase (B. polymyxa, neutral protease, EC 3.4.24.4) was obtained from Boehringer Corporation Ltd. Fluorescein di-acetate and Ouabain were obtained from Sigma Chemicals Ltd. Horseradish peroxidase conjugated Peanut agglutinin (HRP-PNA) was obtained from Miles-Yeda Ltd; Di-phenyl oxazolone (PPO) from International Enzymes Ltd; Triton X-100 was obtained from Baush & Lomb Ltd. Poly-ethylene glycol-2000 (PEG) was obtained from Serva Ltd; Phytohaemagglutinin was obtained from Wellcome Labs Ltd; Agarose was obtained from Calbiochem Ltd. Other chemicals were BDH Analar grade or equivalent.

2:1.2 Media

Glasgow and Dulbecco's modified Eagle's medium were made from 10x concentrated liquid stocks (Flow Labs Ltd; for formulation see current Flow catalogue. The media were diluted with sterile water,

buffered by the adition of IM sodium bicarbonate to give a final concentration of 80mM and supplemented with 10% Newborn Calf serum (EC_{10}) or 10% Foetal Calf serum (EFC_{10}). Sera were obtained from Flow Labs Ltd or Gibco Biocult Ltd. Media were left overnight at 37°C to check for sterility. RPMI 1640 medium was made from powdered medium without bicarbonate or glutamine and buffered by addition of 1M HEPES to a final concentration of 20mM. 1g of sodium bicarbonate was added per litre to yield a final concentration of 10mM and the pH was adjusted to 7.3 by addition of 1M sodium hydroxide prior to filter sterilisation in 5 litre batches, using 0.22µM Millipore filters. The medium was stored at 4°C in 500ml aliquots until it was required, when it was supplemented with 10% Foetal Calf serum and glutamine 20mM final concentration and left overnight at 37°C to test for sterility. No antibiotics were added until after sterility testing.. All media for antibody procedures was supplemented with 10% FCS which had been inactivated by incubation at 56°C for 30 minutes.

2:1.3 Radiochemicals

[^{3}H]-hypoxanthine (1.2Ci/mM), [^{3}H]-uridine (30Ci/mM) and [^{3}H]-thymidine (18Ci/mM) were supplied by Amersham International, Amersham.

2:1.4 Antisera

Monoclonal antibodies F7D5 mouse IgM anti-mouse Thy1.2 ascites fluid (A kind gift of Dr. D.E. Kipp); 11-4.1 mouse IgG2aK anti-mouse H-2K^k ascites fluid (A kind gift of Dr. W. Cushley); 10-3.6 mouse IgG2a anti-mouse IA^k ascites fluid (Kindly donated by Dr. J. Jones); 53-7.313 rat IgG2a anti-mouse Lyt-1 culture supernatant; 53-6.72 rat IgG2a anti-mouse Lyt2 hybridomas were all obtained initially

from the Salk Cell Distribution Centre. Rhodamine conjugated anti mouse whole IgG (Lot S 003); Fluorescein conjugated anti-mouse whole IgG (Lot S 090); Rhodamine conjugated anti-rat whole IgG (Lot S 686); fluorescein conjugated anti-rat whole IgG (Lot S 001) were obtained from Miles-Yeda Ltd.

2:1.5 Cell Culture Lines

The following cell culture lines were used: BHK21/C13 (C13), Syrian baby hamster kidney fibroblasts and TG2, the thio-guanine resistant HGPRT$^-$ mutant (HGPRT , EC 2.4.28) subline of C13 (Littlefield, 1966); BRL, buffalo rat liver epithelial cells (Coon, 1968). L929/A9, the 8-azaguanine resistant HGPRT$^-$ mutant cell line (Littlefield, 1966) derived from L929 (S anford, 1948), a C3H/An mouse embryo fibroblast; HEp-2, a human laryngeal carcinoma cell line (Toolan, 1954); HeLa, a human cervical carcinoma cell line (Gey, 1952); Raji (A), Raji (A)TG, Raji (A)BUdR, an adherent subline and the HGPRT$^-$ and TK$^-$ (TKase, EC 2.7.1.21) derivatives of a human Burkitts B maxillary lymphoma (Pulvertaft, 1964) were gifts of Prof. A.R. Williamson. All other cells were from stocks held by the Wellcome Tissue Culture Unit, Institute of Biochemistry, University of Glasgow, or from the Beatson Institute for Cancer Research, Glasgow.

2:1.6 Salt Solutions and Formol Saline

Balanced salt solution (BSS) and phosphate buffered saline (PBS) were formulated as in 'Cell and Tissue Culture' (Paul, 1979) and formol saline consisted of 10% (v/v), 40% (w/v) Formaldehyde in 0.5% sodium chloride and 1.5% sodium sulphate solution.

2:2 Methods

2:2.1 Maintenance of cell lines

All cells with the exception of L929, L929/A9, Raji and Swiss and BALB/c 3T3 cells were grown in EC_{10} (Glasgow modification) at 37°C in an atmosphere of 95% air, 5% CO_2 in glass Roux bottles or 75cm² tissue culture flasks (Nunc Ltd). L929, L929/A9 and 3T3 cells were grown at 37°C in 5% CO_2, 95% air in Glasgow modified Eagles medium with 10% foetal calf serum (ECF_{10}). Raji and lymphoid cells were grown in RPMI 1640 (Flow Labs Ltd) buffered with 20mM HEPES and supplemented with 10% foetal calf serum, 20mM glutamine and 50μM B-mercaptoethanol in air at 37°C. All cells were subcultured every 3-4 days, diluted 1:10 in the case of fibroblasts and epithelial cell lines, into fresh tissue culture vessels after light trypsinisation (0.25% in 20mM citrate buffer) and pipetting to produce single cell suspensions. Raji cells did not require trypsinisation, only pipetting. Cells were counted using an 'Improved Neubauer' haemocytometer or with a Coulter Counter. Viability was assessed by dye exclusion of 1:1 0.1% Trypan Blue in PBS. Viability was also measured by staining cells with a 9:1 solution of fluorescein diacetate further diluted 1:50 in serum free medium from a stock solution (5mg/ml in Acetone) and incubation for 15 minutes at room temperature. After incubation the cells were exmamined by phase contrast or fluorescence microscopy and counted using an 'Improved Neubauer' haemocytometer.

2:2.2 Lymphocyte Separation

Venous blood was collected by venepuncture into heparinised containers (200 U/ml) and lymphocytes were separated by a modification of the Boyum method (Boyum, 1964). Briefly, the heparinised fresh blood was diluted 1:1 with BSS or serum-free RPMI 1640 (SF-RPMI)

and layered onto Ficoll—Isopaque (ρ = 1.077g/ml^{-1} (Pharmacia Ltd) in the ratio of 2 parts sedimentation medium to 5 parts diluted blood in sterile siliconised 50ml glass centrifugation tubes (Corning Ltd) and centrifuged at 500g for 40 minutes at room temperature. After centrifugation the cell band at the interface was removed and washed four times in large volumes of SF-RPMI to remove platelets and any residual Ficoll-Isopaque. The washed mononuclear cells were resuspended to the initial blood volume in 20% FCS RPMI and incubated at 37°C for 1 hour on glass or plastic to remove monocyte/macrophage cells. The non-adherent cells were removed by shaking and gentle washing with SF-RPMI after incubation. The suspension was washed twice with SF-RPMI and resuspended in 10% FCS complete RPMI. The suspension contained more than 90% lymphocytes estimated by May-Grunwald Giemsa staining and the viability was greater than 95% in all preparations. The adherent cells were shown to be of the monocyte/macrophage lineage using the same staining technique.

2:2.3 [^{3}H]-Uridine Nucleotide Transfer

Freshly trypsinised confluent bulk cultures were transferred at 1x10^5 cells into 50mm tissue culture dishes (5ml/dish) containing 13mm sterile cleaned glass coverslips (Chance Bros Ltd) and incubated overnight. Donor cultures were set up at the same time at 1x10^6 cells in 5ml of EFC$_{10}$ in a 50mm tissue culture dish, left overnight at 37°C and pulsed the next morning for 3 hours with 10µCi of [^{3}H]-uridine per dish. At the end of the labelling period, the labelling medium was removed and the monolayers washed three times with unlabelled medium to remove unincorporated [^{3}H]-uridine. The washed donor cells were then trypsinised and reduced to a single cell suspension before adding to the unlabelled recipients in the dishes

with the coverslips, at a ratio of 1 donor cell to 2 recipients. The donors and recipient cells were co-cultured for 3 hours at 37°C. At the end of the co-culture period the cells were washed three times with BSS to remove any unattached donor cells and fixed with two washes of 10% formol saline of 5 and 15 minutes respectively. The coverslips were then transferred to washing racks and the acid soluble cell material was extracted with 3 washes of excess ice-cold 5% Trichloroacetic acid (TCA) of 15, 5 and 1 minute respectively. The coverslips were then washed with 2 changes of distilled water and dehydrated with one wash of absolute alcohol. After the coverslips had dried they were mounted, cells uppermost, with DEPEX, on glass microscope slides. When dry, the slides were dipped in gelatine chrome alum solution and left to dry. The dry dipped slides were covered with Kodak AR10 stripping film (Safelight, Wratten No. 1) and placed in a light-tight box containing silica gel to dry the stripping film. After 7-10 days exposure the slides were developed in Kodak D19 developer at 20°C for 5 minutes, fixed in a 1:5 dilution of Amfix without hardener (May and Baker Ltd) at 20°C for 4 minutes, washed in tap water for 2 minutes and stained in a 1:20 dilution of Giemsa stain (Gurr & Co. Ltd) for 1 minute. In the case of autoradiographs containing lymphoid cells, a 1:20 dilution of May-Grunwald Stain was added to the diluted Giemsa stain to enhance the staining of the lymphoid cells. After drying the autoradiographs, another 13mm glass coverslip was mounted with DEPEX on top of the film. When dry, the slides were viewed under x100 oil immersion plan objective using a Leitz Orthoplan microscope.

2:2.4 Metabolic Co-operation

Co-cultures of HGPRT$^-$ mutant cells with wild-type cells in a 1:1 ratio were seeded in tissue culture dishes containing glass

resuspended in SF-RPMI, mixed, washed and pelleted by centrifugation at 500g for 5 minutes disgarding the supernatant each time and tapping the tube to disrupt the pellet. The cells were brought to 37°C and 0.8ml of 50% (w/v) PEG-2000 in SF-RPMI at 37°C was added to the final pellet; the mixture was stirred at 37°C for 1 minute then left to stand for 1 minute. At the end of this time, 5ml of SF-RPMI at 37°C was added dropwise over 5 minutes while stirring. Another 5ml of SF-RPMI was added to the cells (while stirring continued), which were then centrifuged at 500g for 10 minutes and washed twice with SF-RPMI to remove the polyethylene glycol. The resultant pellet was resuspended in 40ml of complete RPMI and dispensed into 20 wells of two 24-well plates at 1ml per well. Two wells of each plate were seeded with parental cells at $5x10^5$ cells/ml in 1ml of complete medium and the plates incubated at 37°C overnight. The next day 1 ml of L929/A9 conditioned complete medium, containing $5x10^{-7}$M aminopterin (A), $6x10^{-5}$M, hypoxanthine (H) and $2x10^{-5}$M thymidine was added to each well. The wells were fed every second day by removing 1ml of medium from each well and replacing it with 1ml of fresh conditioned HAT medium, for 4 weeks. The wells were visually checked at feeding for growth, in the wells containing parental and hybrid cells. After selection, the surviving cells were trypsinised, pooled, subcultured and tested for metabolic cooperation, frozen down and stored in liquid nitrogen at the second passage.

2:2.7 Conditioned Medium

Fresh sterile complete medium (200ml) was added to Roux bottles containing confluent L929 or L929/A9 cells for eight hours. The

medium was then removed, centrifuged at 900g for 15 minutes at 4°C, filtered through a 0.22μM Millipore filter and stored in 50ml aliquots at 4°C until required.

2:2.8 Dissociation of the Thymus

Mice of varying ages were sacrificed by cervical dislocation, washed in 70% ethanol (70:30 (v/v) ethanol : distilled H_2O) and the ventral skin removed by making two cuts at sixty degrees to each other under the mandible, to the end of the thoracic cage, peeling back and pinning. The exposed thoracic cage was washed with 70% ethanol to remove any residual hair and an incision was made using sterile scissors, by lifting the clavicle with sterile forceps and cutting, with the points of the scissors pointing upwards, down the sternum from top to bottom of the rib cage. The ribs were folded back and the underlying mesenteries, which anchor the thymus to the rib cage, were cut. The organ was separated from its dorsal mesenteries, taking care to exclude the parathymic lymph nodes lying on either side of the organ. The thymus was removed and held by its vasculature taking care not to disrupt the capsule. The intact thymuses were washed in two changes of SF-RPMI before being minced into 1mm fragments, usually two murine thymuses to 5ml of incomplete medium. The fragments were digested in a 50mm tissue culture dish containing 13mm diameter glass coverslips, to yield sufficient adhering epithelial cells for nucleotide transfer experiments. The fragments were digested using 1000 Units of Hyaluronidase (Fisons Ltd) and 200 Units of Type IV collagenase (Worthington Biochemicals Ltd) for 30 minutes at 37°C. The liberated cells were aspirated from the residue and diluted in 10ml SF-RPMI. The medium was replaced with a further 5ml SF-RPMI containing double the enzyme concentration

and the digestion continued for a further 20 minutes. The liberated cells were again aspirated and diluted, then pooled with the first digest products. The residue was again suspended in 5ml SF-RPMI containing 1000U of hyaluronidase, 200U of collagenase Type IV and 7.5U of Dispase I (Boehringer Ltd). This mixture was incubated for 30 minutes at 37°C, with the residue being pipetted up and down twice during the incubation. The cells liberated into the medium were added to the pooled liberated cells from the two previous rounds of digestion and washed twice in SF-RPMI by centrifuging at 500g for 10 minutes.

2:2.9 Discontinuous Density Centrifugation of Thymus Cells

9ml stock Percoll (poly-vinyl pyrolidone (PVP) coated colloidal silica, Pharmacia Ltd) was made isotonic by the addition of 896.5µl of Hanks (x10) BSS, 45.5µl of 1M HCl and 100µl of 1M HEPES pH7.3 (Ulmer and Flad, 1979). The resulting stock medium has a density of 1.123g/ml and an osmolarity of 301 mOs M. From this stock, solutions of varying density were made using the formula:

ρ = (% stock Percoll x 0.001186) + 1.0041 g/ml

The densities of the resultant solutions were checked by refractive index using an Abbé refractometer. Isotonic Percoll solutions of decreasing density were overlaid in 4x8ml aliquots into 50ml sterile siliconised glass centrifugation tubes and 15ml of thymus cell suspension containing between 2-5x10^6 cells/ml were carefully layered on top of the gradient. The gradients were centrifuged at 600g for 40 minutes at room temperature. The overlying SF medium was aspirated and the resultant bands of cells which had accumulated at the density interfaces were pipetted into SF-RPMI and washed in 20ml of SF-RPMI three times to remove residual Percoll.

2:2.10 Fixation of Acid Soluble Nucleotide Pools with Glutaraldehyde

In an attempt to increase the sensitivity and resolution of the nucleotide transfer technique, cells were labelled in the same manner as previously described (See Section 2:2.3), washed and fixed as before or with varying concentrations (3% - 5%) of glutaraldehyde for varying times (5 - 20 minutes). The fixed cells were processed as before for autoradiography or treated as before to extract the acid soluble and acid insoluble cellular pools, which were then estimated by liquid scintillation counting.

2:2.11 Phytohaemagglutinin (PHA) Stimulation of Peripheral Blood Lymphocytes (PBL)

Peripheral blood lymphocytes (PBL) were separated from freshly drawn heparinised venous blood as previously described (See Section 2:2.2) and stimulated with Phytohaemagglutinin (PHA). PBL suspended in complete RPMI at $1x10^6$ cells/ml were incubated with 10µg PHA/ml of lymphocyte culture at 37°C in humidified air for a maximum of 3 days. The cells were pulsed for 3 hours with 2µCi/ml [^{3}H]-uridine at various times after the addition of PHA to the cultures. The pulsed cells were washed and added to recipient cell cultures to assay for junction formation by measuring [^{3}H]-uridine nucleotide transfer. The cultures were left for 3 hours at 37°C and processed for autoradiography as described before (See Section 2:2.3).

2:2.12 Titration of Complement for Complement Mediated Cytotoxicity (CMC)

Guinea pig serum was prepared from fresh blood collected by cardiac puncture under ether anaesthesia, by allowing the blood to clot at 4°C for 4 hours. The resultant serum was absorbed, with

1g of Agarose (Calbiochem Ltd) to every 5ml of serum for 1 hour at 4°C. The agarose was removed by centrifugation at 1000g for 30 minutes at 4°C. The resultant absorbed serum was divided into 100µl aliquots and rapidly frozen in an acetone/solid carbon dioxide mixture and stored in ampoules at -20°C until required. A freshly extracted single cell suspension of murine thymocytes at $1x10^7$ cells/ml in 10% heat inactivated FCS complete RPMI was plated in 96 well plates in 100µl aliquots. Doubling dilutions of absorbed guinea pig serum were added to the wells in duplicate and the plates incubated at 37°C for 45 minutes. At the end of the incubation 10µl of diluted stock fluorescein diacetate (final concentration 10µg/ml) solution was added to the wells and the cells incubated for a further 15 minutes at room temperature. An aliquot of the cells from each well was viewed under a x25 plan objective by fluorescence and visible light microscopy with a Leitz Orthoplan microscope to assess viability. For each duplicate 200 cells were viewed and the mean percentage of non-viable cells at each dilution was calculated. The highest concentration of guinea pig serum which gave the same percentage viability as the untreated controls was chosen as the working guinea pig serum dilution for CMC.

2:2.13 Titration of Antibodies against Cell Membrane Components.

Using, as before, freshly isolated murine thymocytes as targets for CMC, thymocytes were plated into 96 well plates in 100µl aliquots at $1x10^7$ cells/ml in 10% heat inactivated FCS complete RPMI. Hybridoma supernatants containing specific monoclonal antibodies were used in doubling dilutions in SF-RPMI from neat supernatant. Monoclonal antibodies from ascites fluids were diluted 1:1000 in SF-RPMI prior to doubling dilutions, due to the high titre of antibody in ascites

fluid. The cells were incubated at the appropriate dilutions of antibody for 30 minutes at room temperature. Guinea pig serum was added as a source of complement at the optimal concentration (See Section 2:2.12) and the plates were incubated at 37°C for 45 minutes. After incubation, fluorescein diacetate was added to give a final concentration of 10µg/ml and the cells viewed under phase contrast and epi-fluorescence to determine the percentage of non-viable cells compared with controls.

2:2.14 Detection of Glucose-6-Phosphate Dehydrogenase in Murine/Human Somatic Cell Hybrids (EC 1.1.1.49)

The method used was that of Meera Khan (Meera Khan, 1971). $2x10^7$ cells of the parental cell lines and the somatic cell hybrid were washed twice in 20ml of ice-cold PBS(A) by centrifugation at 500g for 10 minutes. The washed cells were resuspended in 200µl of lysis buffer, which was 5mM phosphate buffer pH6.4, 1mM disodium ethylene diamine tetraacetic acid (EDTA), 1mM B-mercaptoethanol, 0.02mM NADP and held on ice. The cell suspensions were sonicated at 4°C for 30 seconds in micro test tubes and 1ml ice-cold CCl_4 added and emulsified by shaking the capped tube. The emulsion was centrifuged for 25 minutes in a Beckman microfuge (10kg) and the aqueous layer was removed and stored at -70°C until electrophoresis.

Cellulose acetate gel ('Cellogel' Whatmans Ltd) stored in 30% methanol (30% methanol : 70% distilled water) was removed and blotted with fine grain filter paper. The blotted gel was soaked for 10 minutes in the running buffer which was 61.4mM Tris, 4mM EDTA, 13.6mM citric acid pH7.5, blotted once more and washed in two more changes of the running buffer. The gel was once more blotted and applied to a horizontal electrophoresis tank (Shandon Ltd) with an in-between-

the-shoulder gap of 9cm. The circuit was completed with filter paper wicks extending into the running buffer. The cellogel was equilibrated by running at constant amperage, 200v potential difference for 10 minutes. After equilibration 5µl of the aqueous lysate was applied at the cathodal end of the gel, allowed to adsorb and the circuit completed and the electrophoresis run for 2½ hours. The circuit was broken, the gel removed and rolled in the reaction mixture comprising 200µl IM Tris, 4mM EDTA pH8.6, 50µl 0.25M $CoCl_2$, 50µl disodium glucose-6-phosphate (20mg/ml), 50µl NADP (4mg/ml), 50µl 0.25M $CoCl_2$, 50µl disodium glucose-6-phosphate (20mg/ml) and 50µl PMS (0.4mg/ml), adsorbent face down, and left to develop for 30 minutes in a humid atmosphere at 20°C.

2:2.15 Fluorescence Activated Cell Sorting (FACS)

Fresh thymocyte suspensions at a concentration of 10^7 cells/ml were incubated for 30 minutes at 37°C with an excess of the appropriate monoclonal antibody. After incubation the cells were washed once in SF-RPMI at 4°C by centrifugation at 250g for 10 minutes and resuspended in complete RPMI containing 10% heat inactivated FCS to the original concentration at 4°C. The cells were held on ice until FACS analysis was performed. Analysis and sorting were performed on a FACS II (Becton Dickinson Ltd) and the cells illuminated with an argon laser (Spectral Physics Ltd). Prior to analysis, 5µl of 1:10 dilution of fluorescein conjugated anti mouse or rat whole IgG in SF-RPMI was added to the cell suspension and the suspension mixed. The laser was tuned to 488nm giving a power output of 1 watt and a barrier filter of 550nm (Schot Glass Ltd) was placed before the fluorescence detector. The flow rate of the cells was set at 2,000 events per second and 40,000 events were accumulated for analysis.

After analysis, an arbitrary cut off point was chosen, to divide fluorescent from non-fluorescent cells by the distribution of fluorescence in the analysed population. The cells were run again and a minimum of 100,000 cells were collected in each of the channels in 1ml of heat inactivated FCS in 15ml siliconised glass centrifugation tubes (Corning Glass Ltd). The separated cells were washed in SFRPMI by centrifugation at 500g for 10 minutes, resuspended in complete RPMI and used in metabolic co-operation experiments (See Section 2:2.4).

2:2.16 Statistical Analysis of Data

Experimental data were collected and placed in file on the ICL 2976 mainframe computer and subjected to statistical analysis in the MINITAB (Ryan et al, 1981) statistical package. The major statistical test used was the two sample Student's t test, although the Mann-Whitney two sample rank test was used in conjunction with the two sample t test. The major advantages of the two sample t test is that it assumes the two populations are independent and do not have equal variances. The test is more conservative than the pooled t test and is slightly less powerful.

If μ_1, $\overline{x_1}$, s_1 and n_1 are the population mean, the sample mean, the sample standard deviation and sample size for sample 1 and μ_2, $\overline{x_2}$, s_2 and n_2 are the same for sample 2, then the null hypothesis is $\mu_1=\mu_2$ and the alternative is $\mu_1 \neq \mu_2$ and the 95% confidence interval is calculated. As the samples are not assumed to have equal variances the standard deviation of $\overline{x_1}-\overline{x_2}$ is estimated by

$$S = \sqrt{\left(\left(\frac{s_1^2}{n_1}\right)+\left(\frac{s_2^2}{n_2}\right)\right)}$$

and the test is based on the statistic $T = \frac{(\bar{x}_1 - \bar{x}_2)}{S}$

This statistic has approximately a t-distribution with approximate degrees of freedom given by

$$\approx df = \frac{\left(\left(\frac{s_1^2}{n_1}\right) + \left(\frac{s_1^2}{n_2}\right)\right)^2}{\frac{\left(\frac{s_1^2}{n_1}\right)^2}{n_1 - 1} + \frac{\left(\frac{s_2^2}{n_1}\right)^2}{n_2 - 1}}$$

and the null hypothesis i.e $\mu_1 = \mu_2$ rejected for $|t| \geq t$ values of a given (95%) probability with respect to the degrees of freedom in t tables.

The two sample rank test (Mann-Whitney) tests the difference between two population medians and is very powerful if the data are not normally distributed, but assumes that the data are two independent random samples with the same shape and variance. If the samples populations have different shape or different standard deviations, the two sample t test is more appropriate.

CHAPTER 3

RESULTS

'A recognition of the fact that when one is faced with such a multitude of desirable choices, no one choice seems satisfactory for very long by comparison with the aggregate desirability of all the rest, though compared to any one of the rest of the others it would not be found inferior'

John Barth 1958

3:1 Introduction

The phenomenon of metabolite exchange can be used to detect gap junctional communication between cells in culture. The most effective techniques, [^{3}H]-Uridine nucleotide transfer and metabolic co-operation (See Sections 1:2, 2:23, 2:24) have been applied to cells which attach and spread on the substrate. However, lymphocytes attach poorly to plastic or glass, exhibit little spreading and as a result many lymphocytes are lost at the fixation stage of standard nucleotide exchange procedures (See Sections 2:23, 2:24). The cells which do remain attached present difficulties of silver grain counting on autoradiographs, due both to their high grain densities (small area) and the grains not being in the same focal plane caused by the partially flattened shape of the cells even after fixation.

3:1.1 Uridine Nucleotide Transfer between RAJI and BHK/C13 Cells

In the first instance, to overcome the above difficulties an adherent pre-B Burkitts lymphoma line, RAJI, was used in uridine nucleotide transfer experiments, with cells of the baby hamster kidney fibroblast line BHK/C13(C13 cells), which are known to form gap junctions (Pitts, 1971) to test for junctional communication. Both prelabelled, C13 and RAJI cells were used as donors and both cells were used as unlabelled recipients. Silver grains were counted over recipient cells in contact with pre-labelled donors and isolated recipients. Fifty cells were counted in each population, the sample means were calculated and compared statistically using the two sample Student's t test in the statistical package MINITAB (See Section 2:2.16). The data are shown in Table 1.

TABLE 1: URIDINE NUCLEOTIDE TRANSFER BETWEEN RAJI AND BHK/C13 CELLS

n = 50 Mean Grain Count/Cell P > 95% CI

Donor Cells	Recipient Cells	Cells in Contact	Isolated Cells	≈df	t	P
RAJI	RAJI	6.32(3.33)	5.24(3.27)	97	1.63	0.17
RAJI	BHK/C13	12.68(5.73)	12.88	97	0.502	0.617(NS)
C13	RAJI	8.48(3.63)	7.88(4.65)	92	0.72	0.978(NS)
C13	C13	58.32(12.3)	12.32(4.47)	52	21.6	< 0.0001

Donor cells pre-labelled for 3 hours with 2μCi/ml of [^{3}H]-UdR were washed, added at $5x10^4$ cells/plate to $5x10^5$ unlabelled recipient cells, which were established on 13mm sterile glass coverslips overnight in 50mm dishes and co-cultured for 3 hours. At the end of the co-culture period, the cells were fixed with formol saline, processed for autoradiography and the silver grains counted over 50 recipient cells in contact with donors and 50 isolated recipients. Means, standard deviations and degrees of freedom were calculated and these values subjected to two sample Student's t testing. Values of |t| less than the tabulated test statistic at the 95% probability level are classified as non-significant and the null hypothesis, ie equality of means, is not rejected. Values of |t| greater than the test statistic allow the rejection of the null hypothesis and the probability of error (P) is given.

From Table 1, while it can be seen that there is a highly significant statistical difference between C13 recipients in contact with prelabelled C13 donors and isolated C13 recipients, consistant with gap junction mediated transfer. There is no statistical difference in means between isolated recipients and recipients in contact in any combination where RAJI is either donor or recipient. In six attempts of this experiment this observation was consistantly seen, suggesting that if RAJI cells do form junctions, detection is beyond the resolution of this method. The observed result, however, could be due to the length of the co-culture period, which is limited by the half-life of the activity in the nucleotide pool, being too short for detectable gap junction formation to occur.

3:1.2 Metabolic Co-operation between RAJI and BHK/C13-TG2 Cells

To test if increased co-culture time would allow the detection of gap junction formation by metabolite exchange, a series of experiments were performed where RAJI cells were co-cultured in a 1:1 ratio with the HGPRT$^-$ mutant of C13 (TG2 cells) overnight. The cultures are then pulsed with [^{3}H]-hypoxanthine for 3 hours, washed and processed for autoradiography. This method would allow the establishment of gap junctions while not relying on the availability of tritiated label for transfer, as the label is present and available throughout the co-culture, requiring only processing from [^{3}H]-hypoxanthine to [^{3}H]-IMP by the wild-type RAJI cells. TG2 cells will not incorporate significant amounts of hypoxanthine due to the HGPRT defect. To assess gap junction mediated transfer, silver grains over TG2 cells in contact with RAJI cells and isolated TG2 cells, are counted after autoradiography. Discrimination of RAJI cells from TG2 cells is not difficult due to the inability

TABLE 2: SILVER GRAINS OVER TG2 CELLS, ISOLATED OR IN CONTACT WITH RAJI CELLS, LABELLED WITH [^{3}H] HYPOXANTHINE

Grain counts 50 Cells	Mean (SD)	Mean (SD)	≈df	t	P
TG2/RAJI co-culture	TG2/RAJI in contact	Isolated TG2			
	14.44 (7.27)	14.12 (7.31)	97	0.219	0.83(NS)

TG2 and RAJI ($1x10^5$) cells were co-cultured overnight on 13mm glass coverslips in 50mm tissue culture dishes at a 1:1 ratio and pulsed for 3 hours with [^{3}H]-hypoxanthine (2μCi/ml), washed and processed for autoradiography. Grains were counted over 50 TG2 cells in contact with RAJI cells and 50 isolated TG2 cells. Analysis of the grain count data was by the two sample t test.

of the isolated TG2 cells to incorporate the tritiated hypoxanthine during co-culture and the morphological differences between RAJI and TG2 cells. RAJI cells are smaller, less spread on the substrate, exhibit either bipolarity or an attachment point and a spread leading edge and incorporate large amounts of hypoxanthine during co-culture. TG2 cells do not incorporate significant amounts of hypoxanthine and exhibit typical fibroblast morphology with extension of numerous membrane projections at low culture concentrations.

From the data presented in Table 2, a representative experiment in a series of four, it can be seen that there is no statistically significant difference between the mean silver grain count over TG2 cells in contact with RAJI cells in comparison to isolated TG2 cells. During grain counting, care was taken to count only cell pairs which were obviously different in morphology to avoid classifying a RAJI/RAJI pair as a RAJI/TG2 pair and any pair which was morphologically ambiguous was excluded from the sample. These data suggest that RAJI does not form junctions with TG2 cells or that the level of junction formation is too low to be detected by this measure of metabolite exchange. One consequence of the stringent morphological criteria is that large RAJI cells, RAJI cells in S phase, would be excluded from the statistical sample. As a result metabolite exchange experiments with TG2 cells and dextran sulphate mitogenically stimulated RAJI cells were performed.

3:1.3 Uridine Nucleotide Transfer between Dextran Sulphate Stimulated RAJI Cells and TG2 Cells.

To test if any bias was introduced by selecting RAJI/TG2 pairs in the previous metabolic co-operation experiments, the RAJI/TG2 co-cultures were repeated but both RAJI and TG2 cells were used

TABLE 3: RAJI, RAJI(BLAST) AND TG2 URIDINE NUCLEOTIDE TRANSFER EXPERIMENT

Mean Grain Count over Recipient Cells (SD)

Donor	Recipient	Contacting Mean	Non-Contacting Mean	≈df	t	P
TG2	TG2	35.88 (8.28)	3.42 (1.46)	52	26.9	<0.0001
RAJI	RAJI	8.92 (4.29)	7.56 (4.19)	97	1.605	0.11(NS)
RAJI (blast)	RAJI (blast)	13.58 (3.91)	12.7 (4.82)	94	1.003	0.32(NS)
TG2	RAJI	4.52 (2.58)	4.16 (3.11)	94	0.603	0.53(NS)
TG2	RAJI (blast)	4.48 (1.72)	4.24 (2.37)	89	0.58	0.56(NS)
RAJI (blast)	TG2	6.7 (3.2)	6.28 (2.42)	91	0.74	0.46(NS)
RAJI	TG2	5.72 (2.81)	5.44 (3.59)	92	0.453	0.35(NS)

$1.5x10^7$ RAJI cells at $1x10^6$/ml in 150mm plates were exposed to dextran sulphate (2µg/ml) in 15ml of RPMI 1640 for 3 days to produce blast transformation. TG2, RAJI and RAJI(blast) were cultured overnight at $2x10^4$ cells/plate on 13mm glass coverslips as recipient cells. TG2, RAJI and RAJI(blast) donor cells ($1x10^5$ cells/dish) were pulsed with 2µCi/ml [^{3}H]-UdR, washed and co-cultured with the unlabelled recipients. After co-culture the cells were processed for autoradiography and the grains counted over 50 recipients in contact with donors and 50 isolated recipients.

as nucleotide donors by prelabelling with [^{3}H]-uridine. To formally exclude the possibility that RAJI cells in S phase can form junctions with TG2 cells, large RAJI cells in contact with TG2 cells being defined as morphologically ambiguous and omitted from the sample, RAJI cells were stimulated to divide by culture in the B cell mitogen, Dextran Sulphate (Paige, 1978). Three days after the addition of dextran sulphate RAJI cell cultures contained greater than 75% blast cells (S phase) as assessed by May-Grunwald Giemsa staining. These cells and unstimulated RAJI cells were used in uridine nucleotide transfer experiments with TG2 cells and the data are shown in Table 3.

From Table 3 it can be seen that there is no statistically significant difference between TG2 in contact with mitogen treated or untreated RAJI cells and isolated TG2 cells, suggesting that RAJI cells cannot form junctions with TG2 cells or the sensitivity of nucleotide exchange techniques is not sufficiently high to detect low levels of junctional communication betwen RAJI and TG2 cells. This lack of junctional communication may be due to specificity in the cell types involved as seen in BRL cells or by the production of only very transient junctions as seen in V79 cells (Pitts et al, in preparation). The lack of apparent junction mediated communication between RAJI/RAJI pairs logically should exclude the possibility of specificity with respect to the particular cell type interacting with RAJI cells. However, it may be that RAJI cells do not form homotypic junctions and cannot form heterotypic junctions with rodent fibroblasts. To resolve this question nucleotide transfer experiments were performed with rodent, canine and human epithelial cell lines.

TABLE 4: URIDINE NUCLEOTIDE TRANSFER IN RAJI, CK1, BRL AND HeLa CELLS

Grain Counts over Recipient Cells (50 cells mean (SD))

Donor	Recipient	Contacting	Non-Contacting	≈df	t	P
RAJI	RAJI	6.3 (2.15)	5.94 (2.45)	96	0.78	0.43 (NS)
RAJI	CK1	10.33 (4.44)	9.56 (4.78)	96	0.837	0.40 (NS)
RAJI	BRL	4.28 (1.63)	3.96 (2.2)	96	0.826	0.41 (NS)
RAJI	HeLa	7.98 (3.89)	7.32 (3.48)	96	0.894	0.37 (NS)
CK1	RAJI	1.88 (1.51)	1.6 (1.36)	96	0.997	0.33 (NS)
BRL	RAJI	2.1 (1.4)	1.66 (1.19)	95	1.692	0.09 (NS)
HeLa	RAJI	1.86 (1.41)	1.70 (1.43)	95	0.562	0.57 (NS)
CK1	CK1	39.04 (10.4)	4.18 (1.99)	52	23.213	< 0.0001
BRL	BRL	8.76 (4.05)	1.38 (1.07)	55	12.45	< 0.0001
HeLa	HeLa	30.58 (6.51)	1.86 (1.39)	53	30.515	< 0.0001

Donor cells ($1x10^5$ cells/50mm dish in 5ml of RPMI 1640) were pulsed with [^{3}H]-UdR (2μCi/ml) for 3 hours, washed and added to recipient cells ($2x10^4$/50mm dish containing 13mm coverslips). After 3 hours the co-cultures were fixed and processed for autoradiography.

3:1.4 Nucleotide Transfer between RAJI Cells, MDCK1, BRL and HeLa Cells.

Uridine nucleotide transfer experiments were performed using RAJI and canine kidney epithelial cells (MDCK1), buffalo rat liver epithelial cells (BRL) and human cervical epithelial cells (HeLa). MDCK1, BRL and HeLa cells, which all form gap junctions and exchange nucleotides between themselves and with each other, were tested against RAJI cells and the data are shown in Table 4.

From Table 4 it can be seen that gap junction mediated nucleotide transfer cannot be detected between any combination of RAJI cells acting as either donor or recipient. In the case of BRL donors to BRL recipients the reduced grain count in the recipients in contact with donors is due to the formation of 'islands' of BRL cells in the recipient cultures and the rapid spread of the donor label between the individuals cells constituting the 'island', which are all coupled by gap junctions. From these experiments it can be concluded that RAJI cells do not form junctions with this range of epithelial cells or that gap junction formation is a level too low to be detected by this technique.

3:1.5 Metabolite Exchange Experiments between Human Peripheral Blood Lymphocytes (PBL) and RAJI-A(TG) Cells.

While selecting somatic cell hybrids between peripheral blood lymphocytes (PBL) and RAJI cells, Dr G Clements observed that PBL attached to heterokaryons survived much longer than free unbound lymphocytes (Personal Communication). On the basis of this observed phenomenon a series of experiments were devised with PBL and an HGPRT mutant sub-line of RAJI, RAJI-A(TG). If the prolonged survival of PBL in HAT selection medium with heterokaryons was due to metabolite exchange by gap junctions, co-culture of PBL with RAJI-A(TG)

TABLE 5(a): [^{3}H]-HYPOXANTHINE INCORPORATION OF PBL, RAJI(A)T-G AND RAJI-A(TG)/PBL

Co-cultures	Acid Soluble	Acid Insoluble
	(mean cpm (SD) x 10^3)	
RAJI-A(TG)	0.326 (0.0045)	0.862 (0.0063)
PBL	250.6 (22.3)	11.7 (0.6)
RAJI-A(TG)PBL	178.2 (18.9)	10.39 (0.63)

TABLE 5(b): AUTORADIOGRAPHY OF RAJI(A)T-G AND RAJI-A(TG)/PBL CO-CULTURES

Culture	Mean Grain Count/Cell (SD)				
	Cells in Contact	Isolated Cells	≈df	t	P
RAJI-A(TG)	-	4.76 (1.88)	-	-	-
RAJI-A(TG)/PBL	5.88 (2.28)	4.98 (2.94	92	1.711	0.09 (NS)

RAJI(A)TG ($1x10^6$/ml) and PBL ($1.5x10^6$/ml) were cultured alone or at half concentrations in 1:1 culture in 50mm dishes containing 13mm glass coverslips in 5ml of RPMI 1640 in quadruplicate and pulsed with 2μCi/ml of [^{3}H]-hypoxanthine for 3 hours, washed and processed for autoradiography or extracted to yield acid soluble and insoluble fractions (see Methods). RAJI cells with PBL bound were deemed in contact. (Blank 0.0034 (0.0007).)

in the presence of [^{3}H]-hypoxanthine should result in increased silver grains over RAJI-A(TG) in contact with PBL, due to the uptake and conversion of the hypoxanthine by the PBL and the subsequent transfer to and incorporation by RAJI-A(TG) cells.

Experiments were designed to test this possibility by co-culturing PBL and RAJI-A(TG), performing autoradiography on the coverslips and measuring the total incorporation of label into RAJI-A(TG)/PBL co-cultures and RAJI-A(TG) and PBL cultures. From the total label incorporation if no label is transferred by junctions, the co-culture incorporation should be the sum of the individual culture incorporations, but if junctions are formed, the co-culture incorporation should exceed that of the sum of the individual cultures. Label metabolised by the PBL would be available for incorporation in the mutant RAJI-A(TG). As the label is present during the entire coculture period there is no shortage of substrate for the wild-type PBL HGPRT$^+$ to metabolise and thus incorporation into the co-culture should exceed the individual cultures incorporation, even if the uptake of [^{3}H]-hypoxanthine by the PBL is rate limiting, as [^{3}H]hypoxanthine taken up by RAJI-A(TG), while not being metabolised, would be equilibrated with the PBL and available for conversion if gap junctions are formed.

The data from one such experiment are shown in Table 5 (a) and (b) and it can be seen that there is an increase (40%) in the observed incorporation in the mixed culture compared with the expected incorporation from the separate cultures. However from the grain count data (Table 5b), although there is a slight numerical increase in the number of grains over RAJI-A(TG) in the co-culture and RAJIA(TG) cultured alone, it is not statistically significant even given the large sample size of each group.

The expected incorporation for the PBL/RAJI-A(TG) co-culture should be of the order of $125x10^3$ and $6x10^3$ cpm for acid soluble and acid insoluble respectively. The 40% increase in incorporation for both fractions is not reflected in an increased grain count over RAJI-A(TG) as this increased only approximately 15%. The residue of the increased incorporation must be due to increased incorporation into the PBL population in the co-culture, and perhaps greater uptake of hypoxanthine into the acid soluble pool of the RAJI cells, which cannot be converted into acid insoluble material. The other reason for the increase in RAJI-A(TG) cell grain counts could be due to the conversion of the [^{3}H]-hypoxanthine into a product which is not membrane impermeable, but leaks out of the PBL and can be taken up by contiguous RAJI-A(TG) and converted to stable products which remain in the RAJI cells after autoradiographic processing.

From all the experiments performed on RAJI cells no gap junction mediated transfer was detected, but it could not be resolved whether this result was due to lack of gap junction formation or the level of junctional formation was too low to be detected by metabolite exchange. To conclusively answer the question 'Do RAJI cells form gap junctions?', it may be necessary to resort to more sensitive electrophysiological techniques described in Section 1.

3:2 Nucleotide Exchange in PBL, 3T3, TG2 and A9 Cell Co-cultures

From the incorporation data in Table 5, it can be seen that PBL have large pools of labelled material in acid soluble fraction when labelled with [^{3}H]-hypoxanthine. This observation suggests they would be suitable purine nucleotide donors in metabolite exchange experiments if they can communicate by gap junctions. From the published electrophysiological data of Oliveira-Castro and Hulser

TABLE 6: CO-CULTURE OF PHA TREATED AND CONTROL PBL WITH TG2 AND A9 CELLS IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

Mean Grain Count/Cell (SD)

Culture	Cells in Contact	Isolated Cells	≈df	t	P
PBL/TG2	1.66 (1.14)	1.54 (1.01)	96	0.557	0.57 (NS)
4h PHA PBL/TG2	1.92 (1.34)	1.68 (1.08)	98	0.998	0.32 (NS)
24h PHA PBL/TG2	1.92 (1.24)	1.7 (1.11)	96	0.933	0.35 (NS)
48h PHA PBL/TG2	2.48 (1.54)	2.3 (1.33)	98	0.625	0.53 (NS)
72h PHA PBL/TG2	2.36 (1.27)	2.36 (1.21)	97	0.725	0.47 (NS)
* 3T3/TG2	45.7 (5.7)	4.2 (2.2)	52	23.6	< 0.0001
PBL/A9	6.3 (4.03)	6.23 (3.33)	94	0.027	0.97 (NS)

* Pre-labelled [^{3}H]-hypoxanthine nucleotide transfer

Fresh PBL ($1x10^6$/ml) were incubated at 37°C with or without PHA (2µg/ml). After the time indicated, they were washed and added to TG2 ($5x10^4$ cells/50mm dish) on 13mm glass coverslips at $1x10^6$ cells/dish in 5ml of RPMI 1640 containing [^{3}H]-hypoxanthine (2µCi/ml). After 3 hours co-culture the cells were fixed and processed for autoradiography.

* 3T3 cells were pre-labelled with [^{3}H]-hypoxanthine for 3 hours (2µCi/ml) prior to co-culture at $2x10^4$ cells/dish with TG2 cells in the presence of [^{3}H]hypoxanthine. On autoradiography the prelabelled 3T3 cells were easily distinguished by their high silver grain density.

TABLE 7: NUCLEOTIDE TRANSFER BETWEEN 3T3 AND PHA TREATED AND CONTROL PBL

Donor	Recipient	Mean Grain Count/Cell (SD) Cells in Contact	Isolated Cells	≈df	t	P
3T3	3T3	43.28 (13.2)	4.62 (2.41)	52	20.35	< 0.0001
PBL	3T3	2.4 (1.40)	2.24 (1.32)	97	0.588	0.56 (NS)
PBL 4h PHA	3T3	2.34 (1.39)	2.32 (1.2)	95	0.077	0.94 (NS)
PBL 24h PHA	3T3	2.18 (1.12)	2.04 (1.24)	96	0.591	0.56 (NS)
PBL 48h PHA	3T3	2.4 (1.28)	2.3 (1.27)	97	0.393	0.69 (NS)
PBL 72h PHA	3T3	2.88 (1.57)	2.84 (1.4)	96	0.134	0.89 (NS)

Fresh PBL ($1x10^6$/ml) were incubated at 37°C for varying times with PHA (2µg/ml) prior to pre-labelling as donor cells with [^{3}H]hypoxanthine (2µCi/ml) and washed. 3T3 cells ($5x10^6$) were similarly labelled and washed. Donor cells were added to recipient 3T3 ($5x10^5$ cells/50mm dish) on 13mm coverslips at $1x10^6$ PBL and $1x10^5$ 3T3 per dish and cocultured for 3 hours. After co-culture, the dishes were fixed and processed for autoradiography as previously described.

(See Section 1:19), low resistance electrical pathways can be detected only in phytohaemagglutinin (PHA) treated PBL cultures. As a consequence, a series of experiments were designed and performed to utilize the large intracellular acid soluble pool of PBL in the presence of or after prelabelling with [^{3}H]-hypoxanthine. PBL both treated and not treated with PHA were co-cultured with TG2 cells in the presence of [^{3}H]-hypoxanthine, or pre-labelled with [^{3}H]-hypoxanthine and added to cultures of 3T3 cells at times between 4 and 72 hours after addition of PHA to the PBL. The MEC$^-$ cell line A9 was also used in metabolic co-operation experiments with untreated PBL in the presence of [^{3}H]-hypoxanthine. The results of these experiments are shown in Tables 6 and 7.

From these tables it can be seen that neither A9, TG2 nor 3T3 cells in contact with untreated PBL have statistically significant increases from cells not in contact with PBL. It is also shown that TG2 and 3T3 cells in contact with PHA treated PBL do not have significant differences in mean grain counts than TG2 and 3T3 not in contact with PHA treated PBL at all times during the PHA treatment. Nucleotide transfer experiments were also performed using BALB/c3T3, Hep-2 and HeLa cells with PHA treated PBL (data not shown), using the method described in Table 7. In all replicates of these experiments there was always nucleotide transfer in the control cultures, but not in any culture involving PBL either PHA treated or untreated.

There is a number of possible reasons for the disparity between the ionic coupling reports and the metabolite exchange data presented: (1) Gap junction formation does occur in lymphoid cells, but it is of such a low level or for such a brief period that only the highly sensitive technique of electrophysiology can detect it; (2) Lymphoid

cells can form gap junctions, but exhibit a degree of specificity for cell types not seen in any cell phenotype thus far; (3) Lymphocytes cannot form gap junctions and the reported ionic coupling is an artefact caused by close membrane apposition; (4) Lymphocytes do not form gap junctions, but close membrane apposition causes the production of cytoplasmic continuity due to membrane coalescence. The last reason can be dismissed as cytoplasmic bridges would also allow the transfer of nucleotides.

There is considerable evidence that somatic cell hybrids between metabolic co-operation positive (MEC^+) cells and metabolic co-operation negative (MEC^-) cells are MEC^+ and the ability to form gap junctions is dominant in such hybrids (See Hooper and Subak-Sharpe, 1981 for review). Smets et al (1980) remarked that hybrids between MEC^- L929/A9(A9) and PBL were capable of forming gap junctions and that metabolic co-operation and nucleotide exchange were demonstrable. However, no data was presented to substantiate this claim. It was decided that hybrids would be constructed between A9 and PBL.

3:2.1 Somatic Cell Hybrids between A9 and PBL Cells

If the observation of Smets et al is correct, the defect in PBL must be different from that of A9. As a result A9/PBL hybrids were constructed using poly-ethylene glycol fusion. Using PBL and A9 cells, heterokaryons were easily formed, as assessed by phase-contrast microscopy on the basis of differences in the sizes of the two nuclei. However, they grew very slowly, but when A9 conditioned medium was used to support growth in the presence of hypoxanthine aminopterin and thymidine (HAT), the number of colonies which survived four weeks of selection exceeded two hundred, whereas the parental cells all died after 10 days. Rather than attempt to clone the

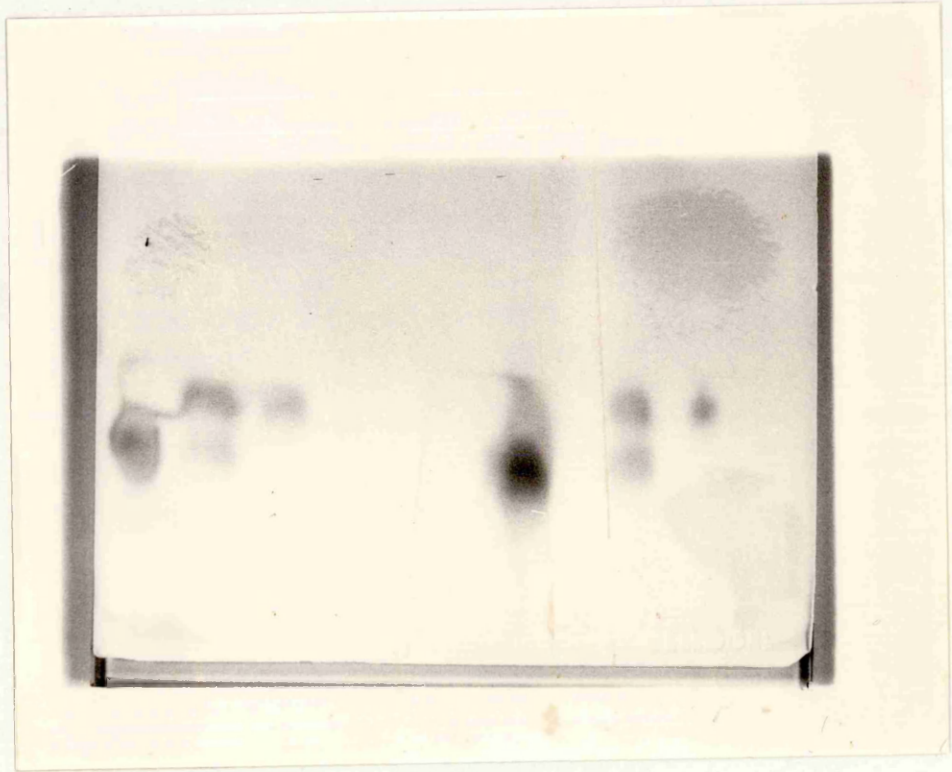
TABLE 8: URIDINE NUCLEOTIDE TRANSFER BETWEEN C13, Hep-2 AND PA

Donor	Recipient	Mean Grain Count/Cell (SD) Cells in Contact	Isolated Cells	≈df	t	P
C13	C13	25.62 (9.12)	2.62 (1.66)	52	17.5	0.0001
C13	Hep-2	23.78 (8.78)	2.08 (1.26)	51	17.3	0.0001
C13	PA	26.44 (11.9)	3.56 (2.36)	52	13.3	0.0001
Hep-2	Hep-2	38.5 (9.07)	7.48 (2.73)	57	23.16	0.0001
Hep-2	C13	44.2 (11 9)	9.02 (4.22)	61	19.7	0.0001
Hep-2	PA	46.36 (17.5)	7.8 (3.34)	52	15.26	0.0001
PA	PA	32.1 (13.3)	4.02 (2.26	51	14.69	0.0001
PA	C13	20.52 (8.6)	2.22 (1.7)	52	14.45	0.0001
PA	Hep-2	26.4 (11.3)	3.14 (2.24)	52	14.33	0.0001

Recipient cells ($5x10^5$) were cultured overnight in 50mm dishes containing 13mm glass coverslips in 5ml EFC_{10}. Donors were pulsed with [^{3}H]-Uridine (2µCi/ml) for 3 hours, washed and $5x10^4$ cells added to dishes containing recipients. The cells were co-cultured for 3 hours, fixed and processed for autoradiography. Silver grains were counted over 50 recipients in contact with donors and 50 recipients not in contact with donors. The means and standard deviations were calculated for both populations and the equality of the sample means tested by two-sided Student's t test.

FIGURE 3

Electrophoresis of glucose-6-phosphate dehydrogenase of PBL/A9 somatic cell hybrids.



surviving colonies, it was decided that the combined selected survivors should be tested for metabolite exchange with C13 and Hep-2 cells. The colonies were pooled and named PA, (P from PBL, A from A9) and the results of these experiments are shown in Table 8.

From the data presented in Table 8 it can be seen that the pooled hybrids can communicate by the exchange of nucleotides between donors and recipients and that this communication is as good as the MEC^+ control cells. Three different methods were used to try and demonstrate the human component of hybrid cells: karyotype analysis, analysis of cell surface expression of human MHC products and isoenzyme analysis. No human chromosomes could be detected by karyotype analysis 6 weeks after fusion. PBL from the human donor were tested for their HLA haplotype; however, when the hybrids and their murine parental line, A9, were tested in complement mediated cytotoxicity (CMC) assay, the rabbit complement alone was cytotoxic to both hybrid and murine parental cells. As a consequence iso-enzymes of the parental and hybrid cells were examined. Glucose 6-phosphate dehydrogenase (EC:1.1.1.49) was chosen as it is present on the X chromosome, which also bears the HGPRT gene. Cells were prepared as described in the Methods Section and supernatants were electrophoresed to separate isotypes. The separated enzymes were exposed to the substrate and the gel is shown in Figure 3. It is evident that the hybrid contains both human and mouse isotypes, but the hybrid isotype could not be visualised. This could be due to inactivity of the hybrid enzymes, preference for the human/human and mouse/mouse sub-unit assembly or the preferential loss of either the human gene in some hybrids and the preferential loss of the mouse gene in others.

As bulk culture for cryo-preservation progressed it was apparent that morphological changes were occurring in cell shape in some of

TABLE 9: URIDINE NUCLEOTIDE TRANSFER BETWEEN PA(f) AND PA(a)

Donor	Recipient	Mean Grain Count/Cell (SD) Cells in Contact	Isolated Cells	≈df	t	P
PA(f)	PA(f)	29.4 (8.0)	2.2 (1.44)	52	23.68	0.0001
PA(f)	PA(a)	17.417 (7.3)	2.75 (2.06)	54	12.351	0.0001
PA(a)	PA(a)	19.34 (11.9)	7.06 (2.98)	55	7.097	0.0001
PA(a)	PA(f)	15.32 (5.28)	1.96 (1.69)	58	17.04	0.0001

Recipient cells ($5x10^5$/50mm dish) were cultured overnight on 13mm glass coverslips in 5m. of EFC_{10} at 37°C. Donor cells ($1x10^5$) were pulsed with [^{3}H]-Uridine (1µCi/ml) for 3 hours, washed and co-cultured with recipient cells for 3 hours. After co-culture, the cells were fixed and processed for autoradiography, with silver grains over 50 recipients in contact with donors and 50 recipients not in contact with donors counted. The grain counts were subjected to two sided students' t test to test for equality of means in the contacting and non-contacting populations.

TABLE 10: URIDINE NUCLEOTIDE TRANSFER BETWEEN PA(6), PA(7), PA(8), PA(9f) AND PA(9a)

		Mean Grain Count/Cell (SD)				
Donor	Recipient	Cells in Contact	Isolated Cells	≈df	t	P
PA(6)	PA(6)	16.75 (4.35)	1.65 (2.06)	27	14.03	0.0001
PA(7)	PA(7)	10.6 (7.57)	2.15 (2.13)	22	4.8	0.0001
PA(8)	PA(8)	24.6 (4.1)	8.1 (3.4)	36	13.82	0.0001
PA(9f)	PA(9f)	29.6 (7.59)	13.2 (3.47)	26	8.76	0.0001
PA(9a)	PA(9a)	22.62 (6.12)	20.14 (5.96)	37	1.33	0.19 (NS)

Legend as for Table 9, except n = 20

the cells. After HAT selection the hybrid cultures exhibited 'fibroblastic' morphology, with 'swirls and whorls' at confluence in contrast to the 'mosaic-like' packing of the A9 parental cells. In dilute culture, A9 cells were smaller, less spread, with little or no membrane projections whereas PA cells were larger, extensive membrane projections and 'astrocyte' morphology. Hybrid cultures at 6 weeks post fusion exhibited, initially, single 'A9-like' cells and later small islands of such cells. However it was noticed that these 'A9-like' islands were less sensitive to the action of trypsin on subculturing allowing comparatively pure populations of the two morphological phenotypes. It was decided to test these two types for metabolite exchange in homotypic and heterotypic combinations.

3:2.2 Nucleotide Transfer between Morphological Types of PA Hybrids

The 'fibroblastic' (PA(f)) and 'A9-like' (PA(α)) morphological types were tested against themselves and each other, to see if shape changes altered their ability to communicate by gap junctions. The data from these experiments are shown in Table 9.

It can be seen from Table 9 that these morphological variations do not affect the ability of the cells to communicate by gap junctions. It is reasonable to assume that the morphology of A9 cells is not a primary cause of its MEC^- characteristic.

3:2.3 Nucleotide Transfer between PA Hybrids of Increasing Age

At every passage the hybrids were tested against themselves to assess their junctional communication by metabolite exchange and data from this series of experiments are shown in Table 10.

From Table 10 it can be seen that as passage number increases, so the MEC^+ phenotype of the cells is lost. This probably reflects the loss of human genetic material from the hybrids as the parental A9 phenotype becomes more dominant in the older cultures, but although the decrease and loss of metabolite exchange is concurrent with the appearance and increase in the PA(a) population, there still exists PA(a) cells at the 9th passage, which could communicate as well as the initially selected hybrids. It is well known that mouse/human hybrids spontaneously lose human genetic material (For review see Ringertz and Savage, 1976). Although mouse/human hybrids are easily constructed, extended culture, cloning and subcloning of individual hybrids would, after as little as four passages, result in the production of predominantly rodent parental cells containing little or no genetic material from the parental human cell increase in the ratio of grains over isolated recipients to recipients in contact, but also in the trend to larger standard deviations, which reflects the increase in recipients in contact with grain counts of the isolated population.

TABLE 11: INCORPORATION OF GLUTARALDEHYDE FIXED OR UNFIXED [^{3}H]-HYPOXANTHINE LABELLED C13 CELLS

Mean (SD) x 10^{-3} CPM

Treatment	Acid Soluble	Filter Bound Acid Insoluble	Total
5% TCA fixed	70.89 (5.03)	3.41 (0.23)	74.3
5' 5% Glutaraldehyde	50.39 (2.4)	7.47 (1.02)	57.86
20' 5% Glutaraldehyde	40.34 (2.44)	8.23 (0.27)	48.58

Four replicate plates containing $2x10^5$ C13 cells were pulsed for 3 hours with 10µCi of [^{3}H]-hypoxanthine in 5ml EC_{10} for each treatment. The plates were washed thrice in BSS to remove unincorporated label and control plates fixed and extracted with 2ml 5% TCA at 4°C. Experimental replicates were fixed with 5% glutaraldehyde for 5 and 20 minutes respectively, then extracted with 2ml 5% TCA at 4°C. All plates were then scraped with a rubber policeman and washed with excess 5% TCA at 4°C onto glass fibre filters. The filters were washed with excess icecold distilled water and dehydrated with absolute alcohol. Glass fibre filters were then incubated at 60°C for 30 minutes with 0.5ml 0.1M hyamine hydroxide, cooled to room temperature, and all samples counted in a Packard 'TriCarb' scintillation counter for four minutes. The mean and standard deviations of the counts per minute of the samples were then calculated. (Blank 32.3 (1.2).)

3:3 Metabolite Exchange and Metabolic Co-operation

The silver grains seen over cells in the final processed autoradiograph are the end-point of a complex multifactorial process. However, they represent the stable products of pathways of which we do have some knowledge and as such reflect gap junction formation and label transfer. Autoradiographic silver grains represent by dint of the processing, the acid insoluble material remaining in the recipient cells, i.e DNA and RNA. The residual material, which ultimately classifies a cell as gap junction competent or not, is by previous measurement only a portion of the radiolabelled material which has been transferred (Pitts and Simms, 1977).

The outcome of the transferred material ultimately resides with the cell into which the radiolabelled material is transferred. If the recipient cell is metabolically active then the label will be converted into stable and unstable material. If given a large enough sample size of a homogeneous (i.e cloned) population, then the incorporation into stable products will be the mean transfer and the mean anabolic activities of the sampled population. However, metabolic co-operation and metabolic exchange could theoretically be measured by the amount of gross exchange of acid soluble and insoluble material. If the acid soluble material could be fixed in recipient cells then reliance on the anabolic state of the recipient cell could be reduced, thus increasing the sensitivity of technique to detect marginal and currently undetectable gap junction formation in mixtures of heterotypic cells. The fixation of acid soluble material was attempted using glutaraldehyde on cells prelabelled with [^{3}H]-hypoxanthine and the results are shown in Table 11.

TABLE 12(a): 'BOOK-KEEPING' FIXATION

Treatment	mean cpm x 10^{-3} 5%TCA	EtOH	5'3%Glut	20'3%Glut	5'6%Glut	20'6%Glut
In fixation agent	-	63.2	83.1	280.4	145.5	297.6
Acid soluble	356.6	313.3	225.6	67.2	183.0	62.9
Filter	91.4	56.7	63.7	64.5	78.5	71.3
Total	448.0	433.2	372.4	412.1	408.0	431.8

TABLE 12(b): GLUTARALDEHYDE QUENCHING

Treatment	BSS + Labelling EC_{10}	6% Glutaraldehyde + Labelling EC_{10}
Mean cpm x 10^{-3}	479.98	388.64

(5 replicates) 19.3% reduction in mean cpm between treatments

(a) Three replicate 5cm dishes containing $1x10^6$ C13 cells in 5ml EC_{10} were pulse labelled for 3 hours with 2µCi/ml [^{3}H]-hypoxanthine, washed with BSS at 4°C and treated with: 2ml of 5% TCA at 4°C for 20 minutes or Absolute alcohol for 20 minutes at 4°C or 3% glutaraldehyde for 5 or 20 minutes or 6% glutaraldehyde for 5 or 20 minutes at 4°C then extracted with 2ml 5% TCA at 4°C except unfixed sample. All samples were then treated as described in Legend to Table 11.

(b) 0.5ml replicates (5) of pooled labelling medium were either adjusted to 6% glutaraldehyde or had an equal volume of BSS added to give identical volumes in both control and glutaraldehyde samples. The replicates were then counted by liquid scintillation counting for four minutes in a Packard 'Tri-Carb' scintillation counter. (Blank 52.5.)

FIGURE 4: HISTOGRAMS OF GRAIN COUNTS OF TCA AND 5 AND 20 MINUTE GLUTARALDEHYDE FIXED C13 CELLS

Legend as for Table 13.

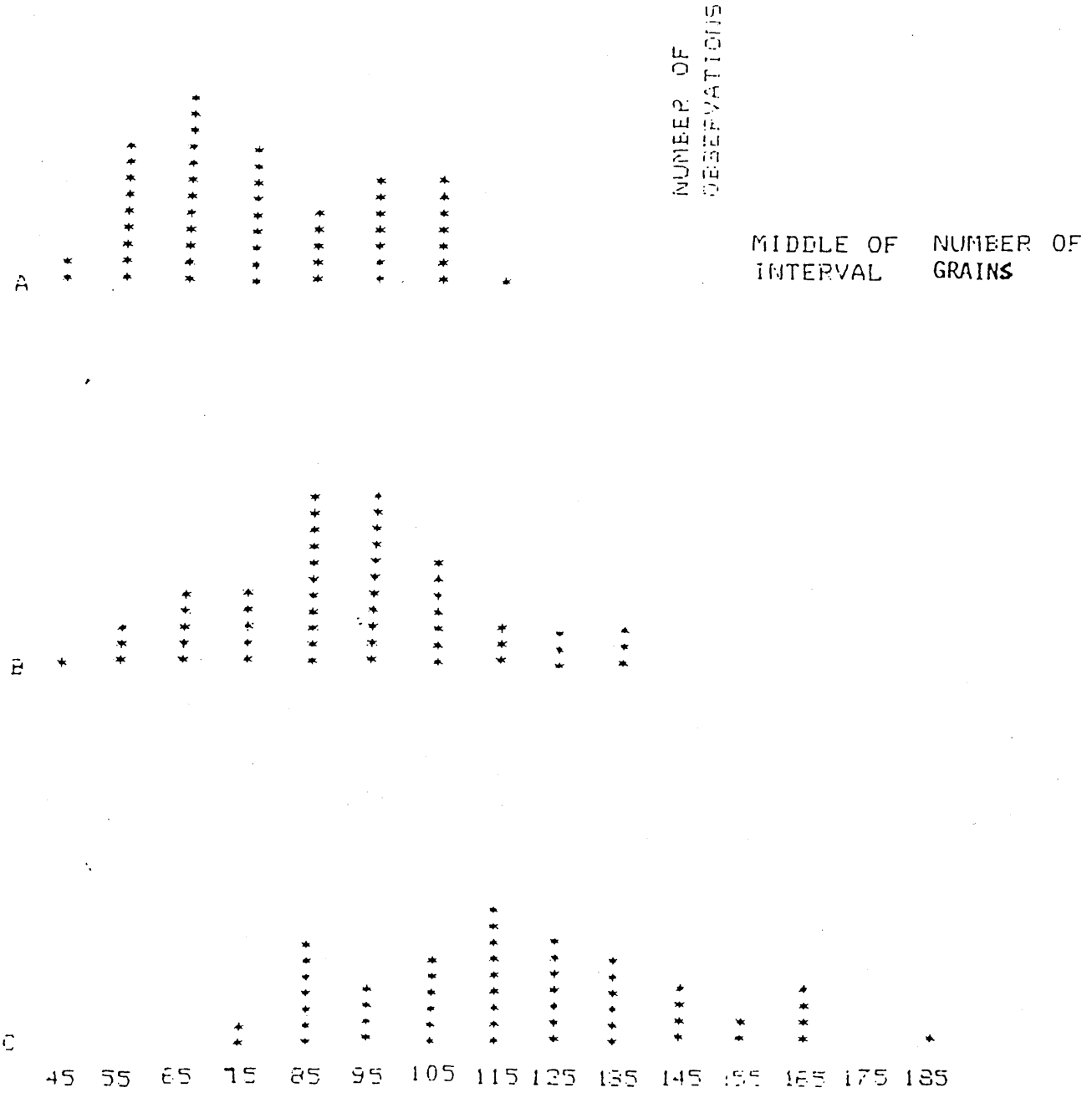


TABLE 13: STUDENT'S t TEST OF GRAIN COUNTS OVER TCA AND GLUTARALDEHYDE FIXED [^{3}H]-HYPOXANTHINE LABELLED C13 CELLS

n = 50

Treatment	mean (SD)	≈df	t	P
TCA fixed	71.64 (18.8)			
		96	3.812	0.0002
5' 5% Glutaraldehyde	87.1 (21.6)			
		94	5.755	0.00001
20' 5% Glutaraldehyde	114.7 (26.1)			
TCA fixed vs 20' 5% Glutaraldehyde		89	9.45	0.00001

$1x10^6$ C13 cells were grown on glass coverslips in 50mm dishes in 5ml EC_{10} and pulsed with 2µCi/ml [^{3}H]-hypoxanthine for 3 hours, washed with BSS and fixed either with 5% TCA (2ml) at 4°C for 20 minutes 5% Glutaraldehyde at 4°C for 5 or 20 minutes. All cells were then extracted with excess ice-cold 5% TCA and the coverslips processed for autoradiography as previously described.

From Table 11 it can be seen that glutaraldehyde only slightly increases the quantity of radioactive material in the acid insoluble fraction, but the quantity of label in the treated samples appears to be reduced. As this was observed in all replicates of this experiment, a 'book-keeping' experiment was designed to examine the apparent loss of label and the results are shown in Table 12(a).

Although there is a marked reduction in the measured incorporation by all glutaraldehyde treated cells the highest acid insoluble radioactivity resides in the TCA fixed samples. Samples which have had exposure to glutaraldehyde all have high release of label into the fixative but high glutaraldehyde concentration and long exposure increase this effect. This accounts for the disparity in total incorporation in treated and control samples in Table 11, but does not account for the reduction in samples fixed with low glutaraldehyde concentration or high concentration for short times. To test if the glutaraldehyde was interfering with the detection of radio-nuclide disintegration, glutaraldehyde was added to labelled medium and counted and the data are shown in Table 12(b).

From Table 12(b) it can be seen that there is considerable 'quenching' in samples containing glutaraldehyde, explaining the abberrations in the 'book-keeping' experiments. When the grain count data from glutaraldehyde treated cells and controls are compared as is shown in Table 13 and Figure 4, it can be seen that there are large statistical differences between the controls and the treated cells. There is a 75% and 15% increase respectively in long and short fixation compared with control cells. When fixation by different agents is considered there is a four-fold greater acid soluble than insoluble pool. In the best junction forming cells, only 10% of the available donor pool is incorporated by the recipient cells (Pitts & Simms, 1977), thus an increase of 75% on glutaraldehyde fixation in

the donor acid insoluble pool would result in a 7•5% increase in the recipient pool. There would be real benefits in sensitivity of metabolite exchange if half the acid soluble pool could have been fixed by treatment with glutaraldehyde, but as the fixed cells were effectively donors not recipient cells, fixation of the acid soluble pool was not as effective as anticipated.

3:4 Metabolite Exchange in Lymphocyte Ontogeny

In order to examine the possible occurrence of gap junction mediated intercellular communication on lymphoid cell ontogeny a series of experiments was devised. The ontogeny of the lymphoid cell series is still poorly understood (see Introduction), but it is generally accepted that the bone marrow is the source of lymphoid cells. However the bone marrow is also the source of all haemopoietic cells in late foetal and adult life, which presents difficulties in differentiating lymphoid cells from other blood cells. Use of this tissue would necessitate overcoming one orother(or both) problems. Either whole marrow populations would have to be used in metabolite exchange, which would present subequent difficulties in identification of the interacting cells. Alternatively the lymphoid elements would have to be separated from the other marrow components. Both these would be difficult and it was decided that primary and secondary lymphoid organs would be a more logical and simpler means of producing lymphoid cells to examine the incidence of possible gap junction formation during lymphoid cell development.

In mammals, the cellular contents of secondary lymphoid organs, lymph nodes, gut associated lymphoid tissue (GALT) and spleen, are to a greater or lesser extent representative of the lymphoid content of blood. The spleen, however, due to its extensive blood supply and functions contains large amounts of non-lymphoid blood cells

in the red pulp. As a result, separation and differentiation of the lymphoid component from the non-lymphoid constituents is difficult. The other secondary lymphoid organs, lymph nodes and GALT, while constituting a large proportion of the total lymphocyte population of the body, are widely disseminated throughout the body's tissues.

For these reasons, it is more logical to consider the primary lymphoid organs as sources of comparatively pure populations of lymphoid cells during differentiation. However, in animals phylogenetically higher than AVES, the Bursa of Fabricus, the organ responsible for the production and maturation of antibody producing B lymphocytes is absent and its function in mammals is thought to have been taken over by the bone marrow.

In mammals the only discernible primary lymphoid organ is the thymus which has a well documented role in the production and differentiation of T lymphocytes (see Introduction). As the thymus consists largely of T lymphocytes and their precursors, further investigations into the possible ability of lymphoid cells to communicate by gap junctions were focused on this organ. Other reasons for this decision were founded on the paucity of non-lymphoid heterogeneity, the ease of preparation of large quantities of T lymphocytes and their precursors, the substantial quantities of published experimental work on the organ and T cell subsets within and arising from this organ and the availability of well characterised monoclonal antibodies against cell surface antigens.

The major restriction hindering the use of human material for these investigations was the difficulty in obtaining suitable human clinical material in a convenient and consistent manner to allow experiments to be carried out on a routine basis. As a result, inbred

TABLE 14: URIDINE NUCLEOTIDE TRANSFER BETWEEN DENSITY SEPARATED THYMOCYTES AND SWISS 3T3 CELLS

n = 50 Mean Grain Count/Cell P > 95% CI

Donor Cells	Recip Cells	Cells in Contact	Isolated Cells	≈df	t	P
3T3	3T3	34.27(3.49)	3.79(1.74)	53	24.83	0.00001
1·055g/ml Thyte	3T3	32.29(10.3)	19.84(7.64)	57	6.78	0.00001
1·065g/ml Thyte	3T3	45.16(12.9)	26.37(7.59)	52	8.98	0.00001
1·075g/ml Thyte	3T3	52.08(10.8)	38.0(11.6)	61	6.41	0.00001
1·090g/ml Thyte	3T3	49.33(11.9)	34.02(9.7)	73	7.17	0.00001
Total Thyte n = 200	3T3	44.89(13.7)	29.72(11.6)	385	12.08	0.00001

$5x10^8$ enzymatically digested washed thymocytes in 20ml SF RPMI were applied to discontinuous isotonic Percoll gradients with steps at 1.055. 1.065, 1.075 and 1.090g/ml density in 50ml siliconised sterile Pyrex centrifugation tubes and spun at 750g for 40 minutes at room temperature. Cells at the interface of the density steps were collected, washed thrice in SF RPMI 1640 and resuspended at $1x10^7$ cells/ml in complete RPMI 1640 containing [^{3}H]-uridine (2µCi/ml) for 3 hours at 37°C. After labelling the cells were washed in complete RPMI and seeded at $1x10^7$ cells onto Swiss 3T3 cells grown overnight at $2x10^5$ cells/50mm dish on 13mm glass coverslips and co-cultured for 3 hours. Similarly prelabelled 3T3 cells were added to identical 3T3 recipients at 5.10^4 cells/dish. At the end of the co-culture the cells were fixed and processed for autoradiography as previously described. Grain counts over 50 recipients in contact with donors and 50 isolated recipients were performed and the means and standard deviations calculated and two sample t tests performed on each group. Donor thyocytes in contact with 3T3 cells were assessed with respect to size and labelling and assigned numerical values 1-6 where 6 was a large heavily labelled donor, 5 large lightly labelled, 4 and 3 medium heavy or light labelled respectively, and 2 and 1 were small heavy or light labelled respectively.

FIGURE 5: HISTOGRAMS OF GRAIN COUNTS OVER ISOLATED 3T3 RECIPIENTS AND RECIPIENTS IN CONTACT WITH DENSITY SEPARATED MURINE THYMOCYTES PRELABELLED WITH [^{3}H]-URIDINE

Legend as for Table 14.

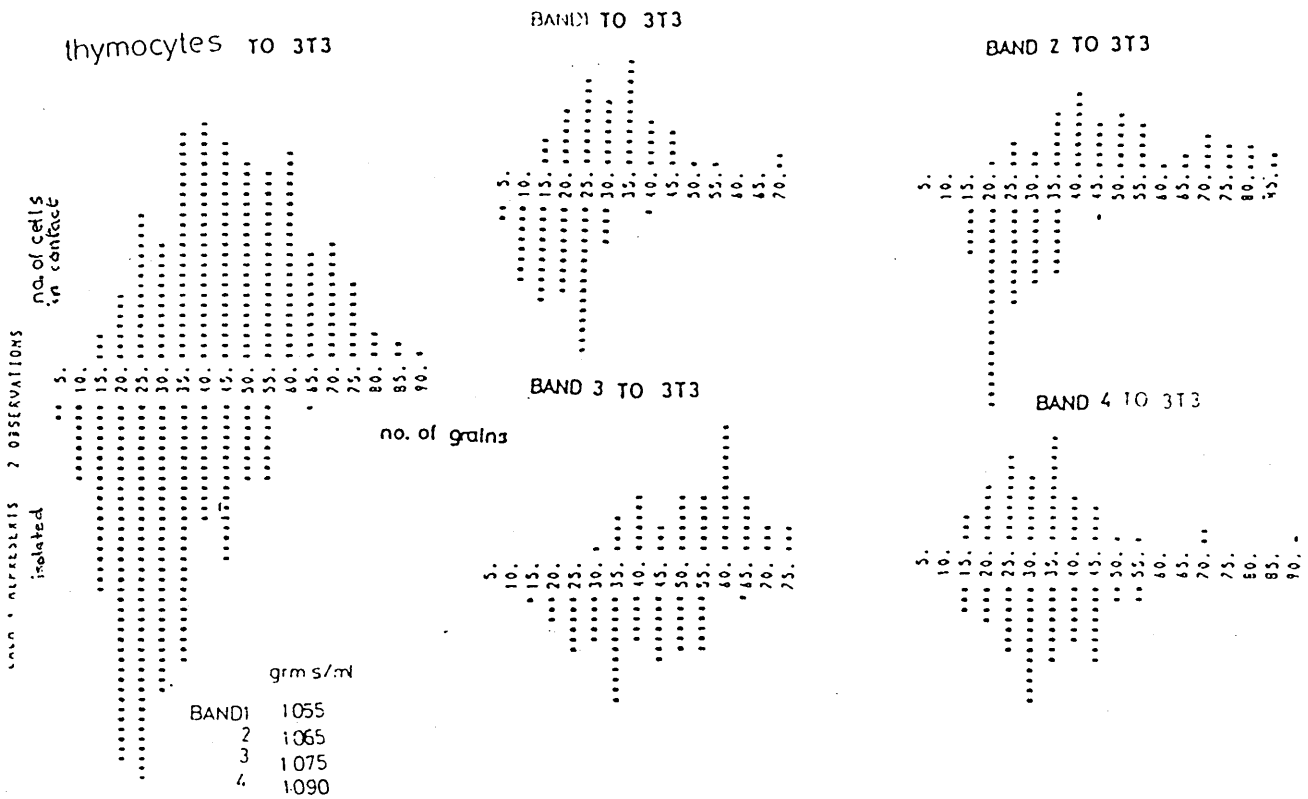
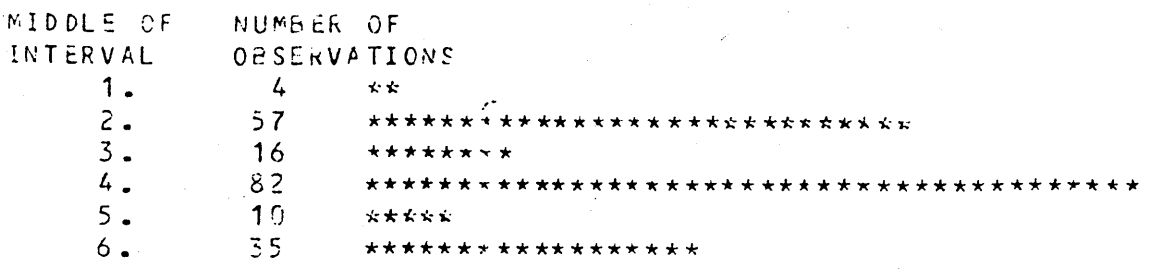


FIGURE 6(b): DISTRIBUTION OF GRAINS OVER LABELLED THYMOCYTES

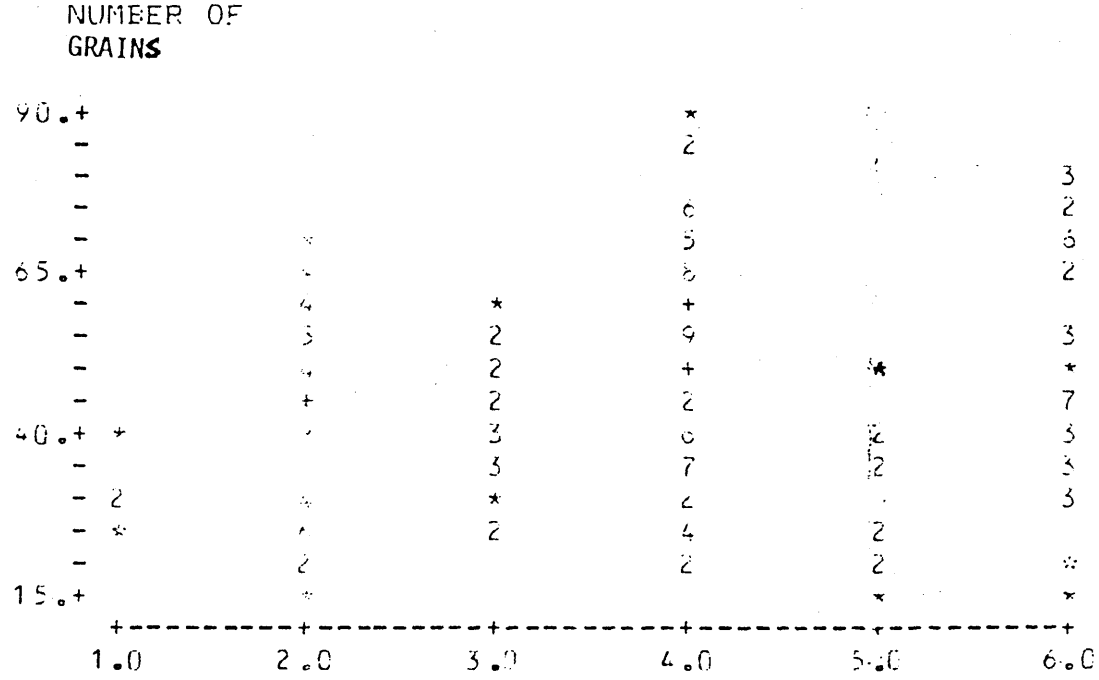
Legend as for Table 14.



Distribution of the number of grains over labelled thymocytes as classified by size and labelling in Table 14.

FIGURE 6(a): GRAPH OF SIZE AND LABELLING OF DONOR THYMOCYTES VERSUS GRAIN COUNTS OVER 3T3 RECIPIENTS IN CONTACT

Legend as for Table 14.



Distribution of grains over recipient 3T3 cells in contact with labelled donor thymocytes classified by size and labelling.

mice were used instead, as they possess well documented cell surface antigens present on thymocytes and T lymphocytes.

3:4.1 Uridine nucleotide transfer between murine thymic lymphocytes and Swiss 3T3 cells

A protocol was devised using mild enzymatic digestion (see Methods) which produced >90% of the constituent cells of the organ, as estimated by microscopic examination of the residue after digestion, with >95% as a suspension of viable single cells as estimated by trypan blue exclusion and fluorescence in the presence of fluorescein di-acetate (data not shown).

In initial experiments using 10^6 thymic lymphocytes per 5cm tissue culture dish in uridine nucleotide transfer experiments, a few recipient Swiss 3T3 cells per coverslip were observed to have greater than background numbers of silver grains when in contact with pre-labelled donor thymocytes. In addition to this observation, it was noticed that these contacting donor cells were large to medium sized. Thus it was decided that the thymic lymphocytes should be sub-divided by their density. The separation of thymocytes of different cellular diameter was achieved by centrifugation at 750g for 40 minutes at room temperature on discontinuous isotonic Percoll gradients with steps at 1.055, 1.065, 1.075 and 1.090g/ml. Cells which banded at the gradient interfaces were washed, readjusted to a concentration of $1x10^7$ cells/ml, pulsed with 10μCi of [^{3}H]-uridine for 3 hours and washed before co-culture with Swiss 3T3 fibroblast recipients. The results of one of these sets of experiments are shown in Figure 5 and the statistical analyses of the total contacting and isolated recipients is shown in Table 14.

TABLE 15: INCORPORATION INTO 3T3 CELLS IN CONTACT WITH OR SEPARATED FROM [^{3}H]-URIDINE PRE-LABELLED THYMOCYTES

	In Contact	Separated	Filters 4 replicates
cpm	mean ± SD	mean ± SD	
Acid Sol	40.7 ± 5.3	141.2 ± 6.3	n.d
Acid Insol	805.5 ± 13.2	47.1 ± 3.1	12.37 ± 24.6

n.d = not done Blank 34.1

$5x10^6$ thymocytes were pulsed for 3 hours with 2µCi/ml [^{3}H]uridine washed 3 times in complete medium (RPMI 1640) and co-cultured with $2x10^5$ Swiss 3T3 cells/5cm tissue culture dish for 3 hours either in direct contact or separated by a 0.22µm Millipore filter. After culture the filter and thymocytes were removed and the 3T3 acid soluble and insoluble pool extracted as previously described. All plates were vigorously washed four times in complete medium and all attached thymocytes removed as judged by phase contrast microscopy in contacting replicates.

The distribution of recipient grain counts plotted against size and incorporation of the donor thymocytes, where group 6 is largest and most heavily labelled and group 1 smallest and most lightly labelled, is shown in Figure 6(a). The distribution of communicating donors by the criteria of size and label is shown in Figure 6(b). From the data in Figure 5 it can be seen from the combined results that the distribution of grain counts over 3T3 cells in contact with thymocytes shows considerable overlap with distribution over the isolated recipients. Many of the 3T3 cells in contact with the added prelabelled thymocytes have a grain count indistinguishable from isolated 3T3 cells, but there is a population which has a higher grain count. Examination of the distributions obtained with the density separated fractions (Figure 5) shows that this property is greatest in Band 2 and these results are confirmed in Table 14 and Figure 6. The occurrence of high grain counts in some 3T3 cells could be due to autoradiograph background or medium transfer of the label. In repeats of this experiment the higher grain count 3T3 cells were always seen regardless of the autoradiographic background.

3:4.2 Analysis of grain counts of 3T3 cells in contact with or separated from labelled murine thymic lymphocytes

The criteria of 'contact' judged by examination with the light microscope is very subjective. Therefore, to test if contact is really necessary to produce the increased grain counts over 3T3 cells, recipient 3T3 cells were separated from prelabelled thymocytes by a 0.22µm Millipore filter. The filter allows free passage of the culture medium but does not allow direct cell/cell contact between donor and recipient cells. Control donor cells were cultured directly with recipient cells and the total incorporation of the test and control recipient cells is shown in Table 15.

As can be seen from Table 15 there is a significant increase in 3T3 recipient incorporation where the prelabelled thymocytes are in direct contact with the recipients. This suggests that while there exists a degree of medium mediated transfer of label from thymocyte to 3T3 cells accounting for the high background in autoradiographs, direct contact is necessary for increased incorporation.

3:4.3 Incorporation of [^{3}H]-Uridine (UdR), [^{3}H]-Thymidine (TdR) and [^{3}H]-Hypoxanthine by thymic lymphocytes

If the sensitivity of the transfer assay could be increased it may be possible to see a larger proportion of thymocyte/3T3 cell pairs showing transfer. Higher activity in the acid soluble pool should result in greater transfer of activity. Other precursors were therefore examined to see if they gave a higher nucleotide (acid soluble) pool activity and the incorporation and grain counts are shown in Table 16.

From the incorporation data it is evident that although hypoxanthine has a sixfold lower specific activity than [^{3}H]-UdR, it has an almost fourfold increase in the acid insoluble pool and a comparable acid soluble pool. This increase in incorporation is also mirrored in an almost 2.5-fold increase in grain counts after autoradiography of cytocentrifuge preparations. The uptake of each of the labels is comparable when comparing the acid soluble pools, with the acid insoluble incorporation of [^{3}H]-TdR giving a measure of DNA synthesis.

TABLE 16: INCORPORATION OF THYMIC LYMPHOCYTES OF [^{3}H]-THYMIDINE (TdR), [^{3}H]-URIDINE (UdR) AND [^{3}H]-HYPOXANTHINE BY LIQUID SCINTILLATION COUNTING AND AUTORADIOGRAPHIC SILVER GRAIN COUNTING

	Mean cpm (SD) 4 replicates		
	[^{3}H]-TdR	[^{3}H]-UdR	[^{3}H]-Hypoxanthine (HX)
Acid soluble	535,895 (4,597.5)	612,990 (3,522.7)	645,614 (1917.2)
Acid insoluble	7,294 (204.2)	2,568 (124.2)	9212.7 (198.4)
Mean Grain Counts (SD) n = 50	2.37 (1.97)	11.34 (8.19)	29.84 (6.34)
Specific Activity (Ci/mM)	25	28	5

10^7 thymocytes pulsed for 3 hours with 10μCi of label, washed thrice in complete RPMI, cytocentrifuge preparations of aliquots processed for autoradiography. Acid soluble pools extracted with 2x2ml of ice-cold 5% TCA, pooled and counted; acid insoluble fractions extracted with 2ml 0.1M NaOH at 20°C for 30 minutes and acidified with 0.3ml 1MHCl and counted. The specific activities cited are the Amersham International quoted specific activities.

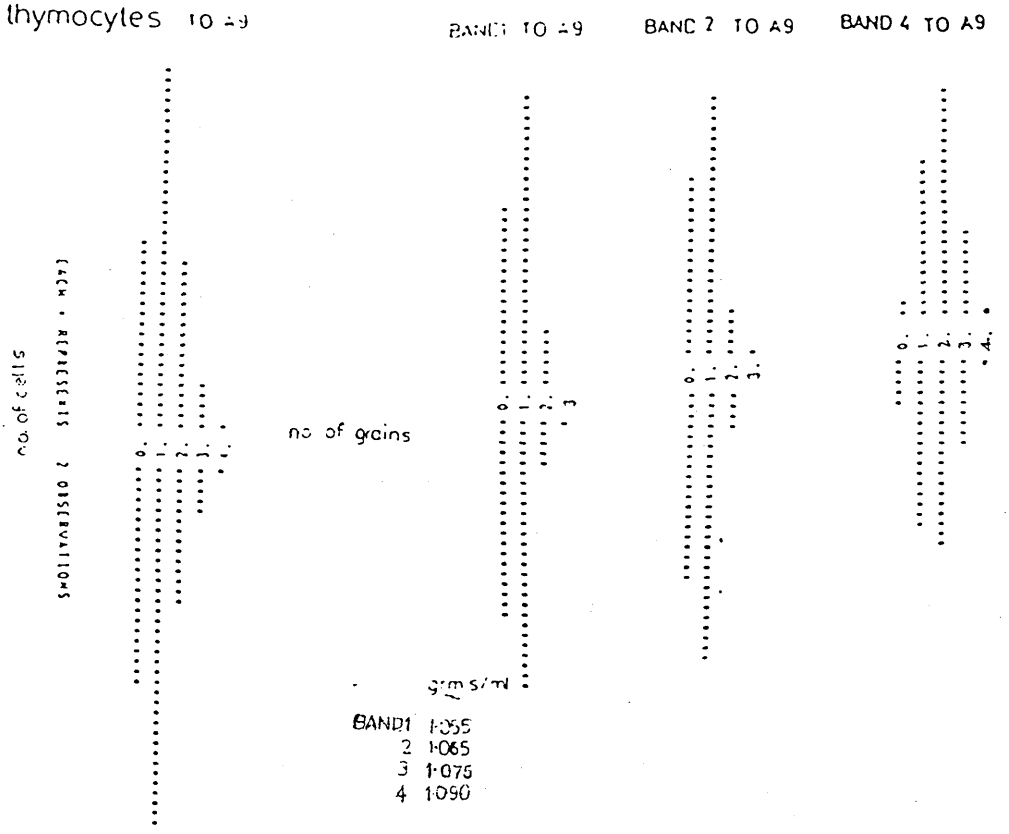
TABLE 17: METABOLITE EXCHANGE BETWEEN DENSITY SEPARATED THYMIC LYMPHOCYTES AND TG2 OR A9 CELLS IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

Culture	Mean Grain Count/Cell (SD) Cells in Contact	Isolated Cells	≈df	t	P
Band 1(1.055g/ml) Thymocytes/A9	0.774(0.64)	0.755(0.705)	93	0.144	0.89(NS)
Band 2(1.065g/ml) Thymocytes/A9	0.78(0.078)	0.7(0.614)	93	0.603	0.55(NS)
Band 4(1.090g/ml) Thymocytes/A9	1.824(0.842)	1.686(0.948)	98	0.773	0.44(NS)
Thymocytes/TG2	28.981(6.66)	10.577(4.27)	86	16.76	<0.0001

$1x10^7$ thymocytes separated by density centrifugation (as previously described) for A9 experiments (densities 1.055, 1.065, 1.090 respectively) and unseparated for TG2 cells, were cocultured with $2x10^5$ A9 or TG2 which had been cultured on 13mm coverslips in 50mm dishes overnight. Cells were co-cultured in the presence of [^{3}H]-hypoxanthine (50µCi) in 5ml of complete RPMI for 3 hours. The plates were washed after co-culture, fixed and processed for autoradiography as previously described. 50 isolated recipients and 50 recipients in contact with thymocytes were grain counted.

FIGURE 7(a): HISTOGRAMS OF GRAIN COUNTS OVER ISOLATED A9 AND TG2 RECIPIENTS AND RECIPIENT CELLS IN CONTACT WITH THYMOCYTES IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

Legend as for Table 17.



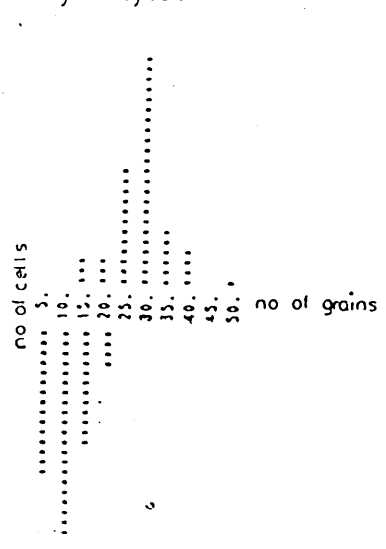


FIGURE 7(b): DISTRIBUTION OF SIZE AND LABELLING OF THYMOCYTES IN CONTACT WITH TG2 CELLS CO-CULTURED IN THE PRESENCE OF [^{3}H]HYPOXANTHINE

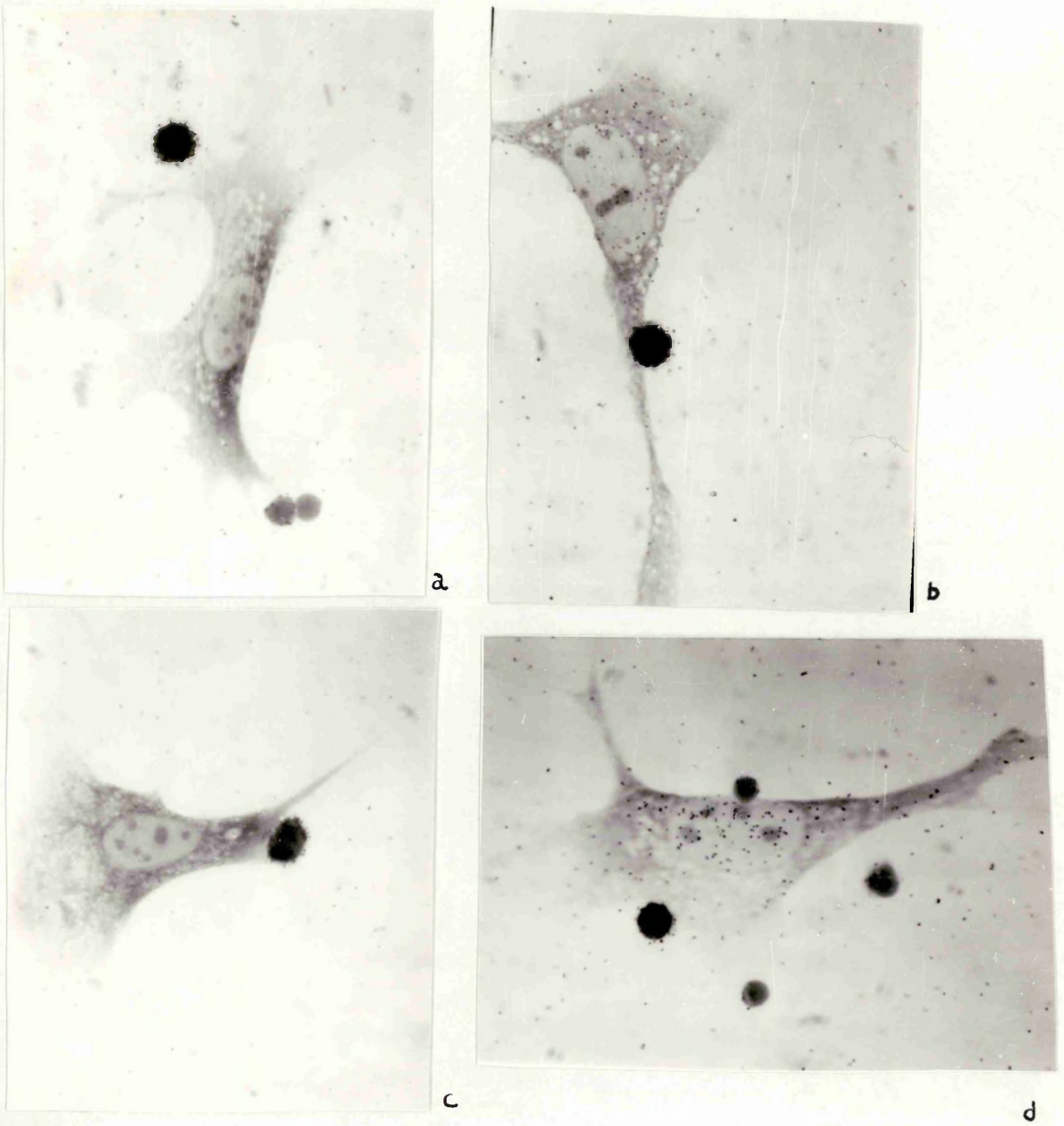
Legend as for Table 17.

MIDDLE OF INTERVAL	NUMBER OF OBSERVATIONS	
1.	6	******
2.	13	*************
3.	4	****
4.	19	*******************
5.	2	**
6.	8	********

Distribution of labelling of thymocytes in contact with TG2 recipients.

FIGURE 7(c): EXAMPLES OF CELLS AFTER AUTORADIOGRAPHIC PROCESSING

Legend as for Table 17.



(a) + (b) 1.065g/ml thymocytes in contact with TG2 recipients;

(c) + (d) 1.090g/ml thymocytes in contact with Tg2 recipients.

3:4.4 Metabolic co-operation between thymic lymphocytes and TG2 and A9 cells

The incorporation of label in the preceeding experiment reflects the level of labelling that would be achieved in a metabolic co-operation experiment using thymocytes in the presence of [^{3}H]-hypoxanthine. Given the above data it was decided that metabolite exchange experiments would be attempted to test the ability of thymic lymphocytes in this more rapid assay using the HGPRT$^-$ mutant of BHK/C13, TG2 and the HGPRT$^-$ mutant L929, A9. The important difference between these lines is that the hamster TG2 line is MEC$^+$ and the mouse A9 is MEC$^-$ and neither incorporate [^{3}H]-hypoxanthine. TG2 when co-cultured with wild-type MEC$^+$ cells will incorporate [^{3}H]-hypoxanthine derived nucleotides produced in the wild-type cells by transfer through gap junctional channels. TG2 cells growing in contact with HGPRT$^+$ MEC$^+$ cells therefore become labelled in the presence of [^{3}H]-hypoxanthine whereas A9 cells do not.

Thymic lymphocytes (10^7) were cultured in media containing 50μCi of [^{3}H]-hypoxanthine in the presence of either TG2 or A9 cells. The results of these experiments are shown in Figure 7(a) and (b) and the statistical analyses are shown in Table 17. The data from these experiments show that some of the MEC$^+$ TG2 cells in contact with thymocytes incorporate labelled material whereas none of the A9 MEC$^-$ cells in contact with thymocytes is labelled above background levels.

From the distribution of the donor adherent thymocytes in the TG2 co-culture experiment in Figure 7(b) it can be seen that medium

and large thymocytes outnumber small lymphocytes by more than two to one. There is, as in the experiments with 3T3 cells, an overlap in grain count distribution of TG2 cells in contact with the distribution of isolated TG2 cells.

3:4.5 Analysis of adherence of thymic lymphocytes to homologous or heterologous stromal substrates

In order to examine the conditions which give rise to the overlapping distributions of grain counts between contacting and non-contacting recipients some consideration must be made of the events which result in the transfer of the labelled material between donor and recipient cells. The cellular events which result in the transfer of material by gap junctional channels can be largely summarised by regarding the process as four steps from the addition of the donor cells to the co-culture. The cells must settle, adhere, form junctional channels and transfer the label. This is a grossly simplified model of a highly complex process within which three of the four steps cannot be resolved. The process of cell settling, junctional channel formation and label transfer are parameters which would prove difficult to measure using the protocols of metabolite transfer. However, adhesion is a parameter which can be quantified using previously described techniques, as the number of adherent cells can be measured after incubation, fixation and staining and this proportion measured with respect to the total input of cells at the beginning of the experiment.

The basic protocol was to take male and female weanling (3-4 week old) mice from sibling congenic CBA mothers, half of whom were used on day 1 to set up replicate cultures of thymic stroma. On day 2, the stromal cultures were washed and briefly treated with

TABLE 18: ADHESION OF THYMIC LYMPHOCYTES TO STROMAL CELL CULTURES

n = 20 mean (SD)

Thymocytes	Stroma	Thymocytes/Unit Area	Thymocytes/Stromal cell	
Male	Male	306.4 (167)	10.32 (6.23)	1
Male	Female	365.8 (205)	11.61 (6.27)	2
Female	Female	250.2 (138)	7.4 (5.77)	3
Female	Male	273.7 (143)	8.03 (5.12)	4

Two sided t test

Density	n	≈df	t	P
1 vs 2	20	36	-1.005	0.32 (NS)
3 vs 4	20	37	-0.529	0.59 (NS)
Bound				
1 vs 2	38	73	-0.900	0.37 (NS)
3 vs 4	38	72	0.505	0.61 (NS)

Randomly selected male and female 3-4 week weanling CBA congenic mice were chosen, half of whom were used on day 1 to produce thymic stromal cell cultures. Thymuses were removed and dissociated using the enzymatic digestion described in the Methods section, washed in SF RPMI and placed in 50mm dishes at $5x10^7$ cells/dish in 5ml of complete RPMI. The plates were incubated at 37°C for 6 hours, washed four times with complete RPMI after incubation and the medium replaced and the residual cells incubated at 37°C overnight. On day 2, the plates were washed and briefly treated with 0.25% trypsin solution to remove residual lymphocytes and the medium replaced. The remainder of the male and female mice were sacrificed, thymuses removed and enzymatically dissociated into single cell suspensions. $1x10^7$ thymocytes were added to the stromal cultures in all possible combinations and incubated at 37°C for 3 hours. After coculture the plates were fixed and stained with May-Grunwald-Giemsa-Rhodamine B as described in the Methods section. The cultures were quantitated by two parameters, number of thymic lymphocytes bound to 38 stromal

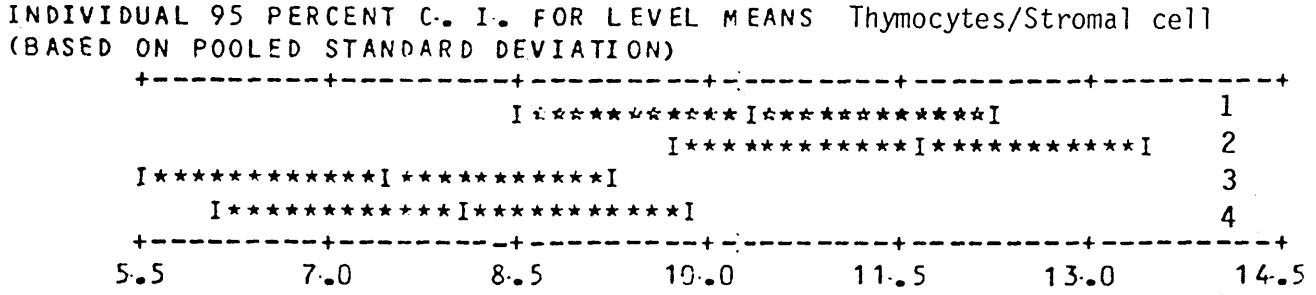
TABLE 18: Legend, cont'd ...

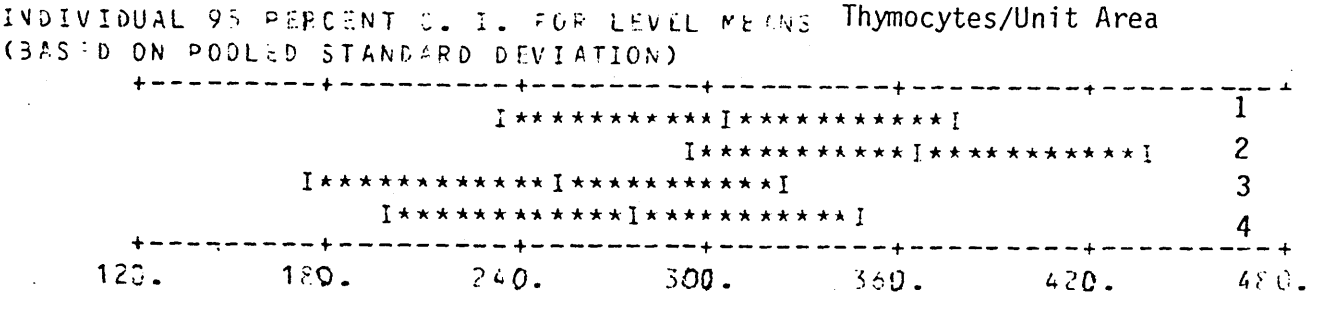
cells chosen at random and the number of thymic lymphocytes in 20 areas (0.5mm x 0.5mm) at random on the 50mm dishes. The defined area was established by viewing an 'Improved Neubauer' haemocytometer under a x40 plan objective and projecting a 0.5mm x 0.5mm square onto a television monitor screen via a video camera connected to the microscope.

FIGURE 8: GRAPHICALLY PLOTTED 95% CONFIDENCE INTERVALS FOR LEVEL MEANS OF THYMIC LYMPHOCYTES BOUND TO STROMAL CELLS AND NUMBER OF THYMIC LYMPHOCYTES IN A 0.5mm x 0.5mm AREA

Legend as for Table 18.

Plotted individual 95% confidence interval for level means based on pooled standard deviation for number of thymic lymphocytes bound to stromal thymic cells (Bound) and the number of thymic lymphocytes per $0.25mm^2$. Central bar indicates the location of the mean and outer bars define the calculated 95% confidence interval of the calculated mean.





trypsin to remove any residual lymphocytes and the medium replaced. The remaining male and female weanlings were used to produce single cell suspensions of thymic lymphocytes and 10^7 lymphocytes were added each possible combination to the replicate male or female stromal cell cultures and incubated for 3 hours, the length of time of the co-culture in the metabolite exchange experiments. After co-culture the plates were fixed and stained in the manner previously described. The cultures were quantitated using two different parameters, the density of adherent cells, and the number of cells bound to a given number of individual stromal cells. The density was estimated by microscopically viewing 20 (0.5mm x 0.5mm) defined areas on the 5cm tissue culture plates. The defined area was set by viewing an 'Improved Neubauer' haemocytometer under x40 plan objective and projecting a 0.5mm x 0.5mm square onto a television screen via a video camera mounted on the microscope then viewing the area and counting the total number of cells bound in the area and the number of thymocytes bound to stromal cells. The data from one of this series of experiments are shown in Table 18 and Figure 8.

From this data it can be seen that male thymocytes show higher binding to glass and stromal cells, but there is more adhesion in heterotypic cell combinations with both male and female thymocytes. From the 95% Confidence Intervals of the mean thymocytes bound to stromal cells (Figure 8) it can be seen that there is a large range of thymocytes bound. From the raw data (not shown) two populations of thymocyte binding to stroma can be discerned: one which has less than ten thymocytes per stromal cell and other stromal cells with up to 28 thymocytes bound. Whether these two populations are real or merely experimental artefacts could not be determined as the stromal cells show enormous variation in size, spreading and

TABLE 19 : METABOLIC CO-OPERATION OF THYMIC LYMPHOCYTES OF MICE OF DIFFERENT AGES AND TG2 CELLS

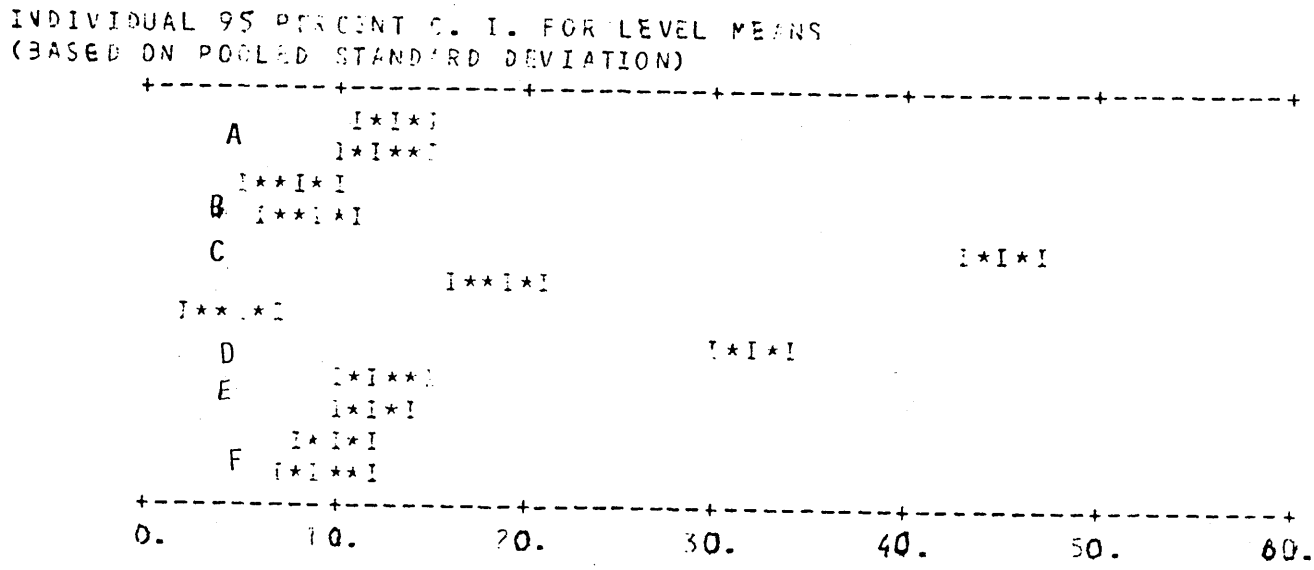
Mean grain count/cell n = 50

	Co-Culture	Cells in Contact	Cells	≈df	t	P
A	TG2.2 wks gestn	13.06 (4.03)	12.38 (4.96)	94	0.752	0.45 (NS)
B	TG2/new born	8.76 (3.01)	7.66 (3.77)	93	1.612	0.11 (NS)
C	3 wks/TG2	45.0 (23.61)	18.72 (8.48)	61	7.407	0.00001
D	6 wks/TG2	31.96 (10.49)	4.78 (1.36)	50	18.166	0.00001
E	12 wks/TG2	12.28 (3.42)	12.1 (3.52)	98	0.259	0.79 (NS)
F	Adult/TG2	9.86 (4.10)	9.42 (4.24)	95	0.63	0.52 (NS)

Congenic CBA mouse thymuses of different ages were digested as before to single cell suspensions, washed and co-cultured with TG2 cells ($1x10^5$/dish) in 50mm dishes on 13mm coverslips, at $2x10^6$ thymocytes/dish in the presence of 50µCi of [^{3}H]-hypoxanthine in 5ml complete RPMI 1640. After co-culture the cells were fixed and processed for autoradiography as previously described. Grains were counted over 50 TG2 cells in contact with thymocytes and 50 TG2 cells not in contact. The means and standard deviations were calculated and subjected to two-sample t tests to test equality of means.

FIGURE 9: GRAPHS OF 95% CONFIDENCE INTERVAL OF SAMPLE MEANS VERSUS GRAIN COUNTS OF METABOLIC CO-OPERATION OF TG2 CELLS AND THYMOCYTES FROM MICE OF DIFFERENT AGES IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

Legend as for Figure 8 and Table 19.



Distribution of grains over recipients in contact and isolated recipients of mice of different ages as described in Table 19.

morphology within high and low binding stromal cells. It may well be that all stromal cells bind equal numbers of thymocytes, but bind with different strengths, the loosely bound thymocytes being lost during subsequent processing.

3:4.6 Metabolic co-operation of TG2 and thymocytes of mice of different ages

In all experiments thus far, the mice used were weanlings; also mice of this age show rapid increases in thymic size and cellular content and were thought a good source of lymphocytes at different stages of differentiation. It appears that there is a small proportion of MEC^+ lymphocytes in weanling mouse thymuses, but there could be a greater proportion in mice of different ages. Two weeks gestation, new born, 3 week, 6 week, 12 week and mature adult mouse thymocytes were tested in metabolic co-operation with TG2 cells in the presence of [^{3}H]-hypoxanthine. The results of a representative sample of experiments are shown in Figure 9 and Table 19.

It can be seen from the table that metabolite transfer only occurs with sufficient frequency to generate statistically significant increase in the average grain counts of TG2 cells in contact with thymocytes of 3 to 6 week old mice.

However, it is possible to record individual TG2 cells in contact with thymocytes which have a grain count well above background between 3 and 6 weeks post-nativity. On scanning 3 replicate coverslips for each time point in thymic development a single example of metabolite exchange was seen with the neonatal thymocytes and two examples seen with foetal thymocytes.

A variety of outbred and inbred mouse strains were tested as weanlings, all of whom exhibited similar and comparable metabolic co-operation to CBA mice previously extensively tested. No exceptions

TABLE 20: METABOLIC CO-OPERATION OF TG2 CELLS AND THYMOCYTES AFTER IN VITRO CULTURE IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

Co-culture	Mean grain counts/cell (SD) n = 50 Cells in Contact	Isolated Cells	≈df	t	P
TG2/Day 0 Thytes	22.6 (14.4)	3.74 (3.68)	55	0.004	0.00001
TG2/Day 1 Thytes	3.36 (2.12)	3.64 (2.14	97	-0.658	0.51 (NS)
TG2/Day 2 Thytes	3.78 (2.51)	3.78 (2.51)	98	0.00	1.0 (NS)

Six CBA sibling weanling mice (4 wks) were sacrificed, the thymuses were removed and enzymatically digested, as described before, to a single cell suspension. The thymic lymphocytes were washed and an aliquot ($5x10^6$ cells) was co-cultured with $2x10^5$ TG2 cells on 13mm coverslips in 50mm dishes in the presence of 50μCi of [^{3}H]-hypoxanthine for 3 hours, in 5ml complete RPMI. After co-culture the cells were processed for autoradiography. The majority of the cells were placed in culture in 15cm plates at $2x10^7$ cells/ml in complete RPMI overnight at 37°C and 5% CO_2/95% air in a humidified incubator. The next day the cells were pooled and dishes washed with excess sterile SF-RPMI. The viability was assessed by fluorescence of cells after incubation of cells in fluorescein di-acetate and the cell concentration readjusted to $2x10^6$ cells/ml in SF-RPMI. Cells in SF-RPMI were applied to Ficoll cushions of 1.077g/ml and spun at 500g for 20 minutes to remove dead cells. The cells at the interface of the RPMI and Ficoll were harvested and washed twice in SF-RPMI. An aliquot was removed and tested for metabolic cooperation as on Day 0. The rest of the cells were returned to a concentration of $2x10^7$/ml and culture as before, overnight. The same procedure was repeated the next day. The mean viabilities and (SD) on Day 0, Day 1 and Day 2 were: 96.4 (7.1); 53.8 (12.3); 81.4 (6.1) respectively, as calculated from viabilities assessed after overnight culture not as total viability from starting viability.

FIGURE 10(a): HISTOGRAMS OF TG2 CELL GRAIN COUNTS IN CONTACT WITH OR ISOLATED FROM DAY 0, DAY 1 AND DAY 2 IN VITRO CULTURED WEANLING CBA THYMOCYTES.

Legend as for Table 20.

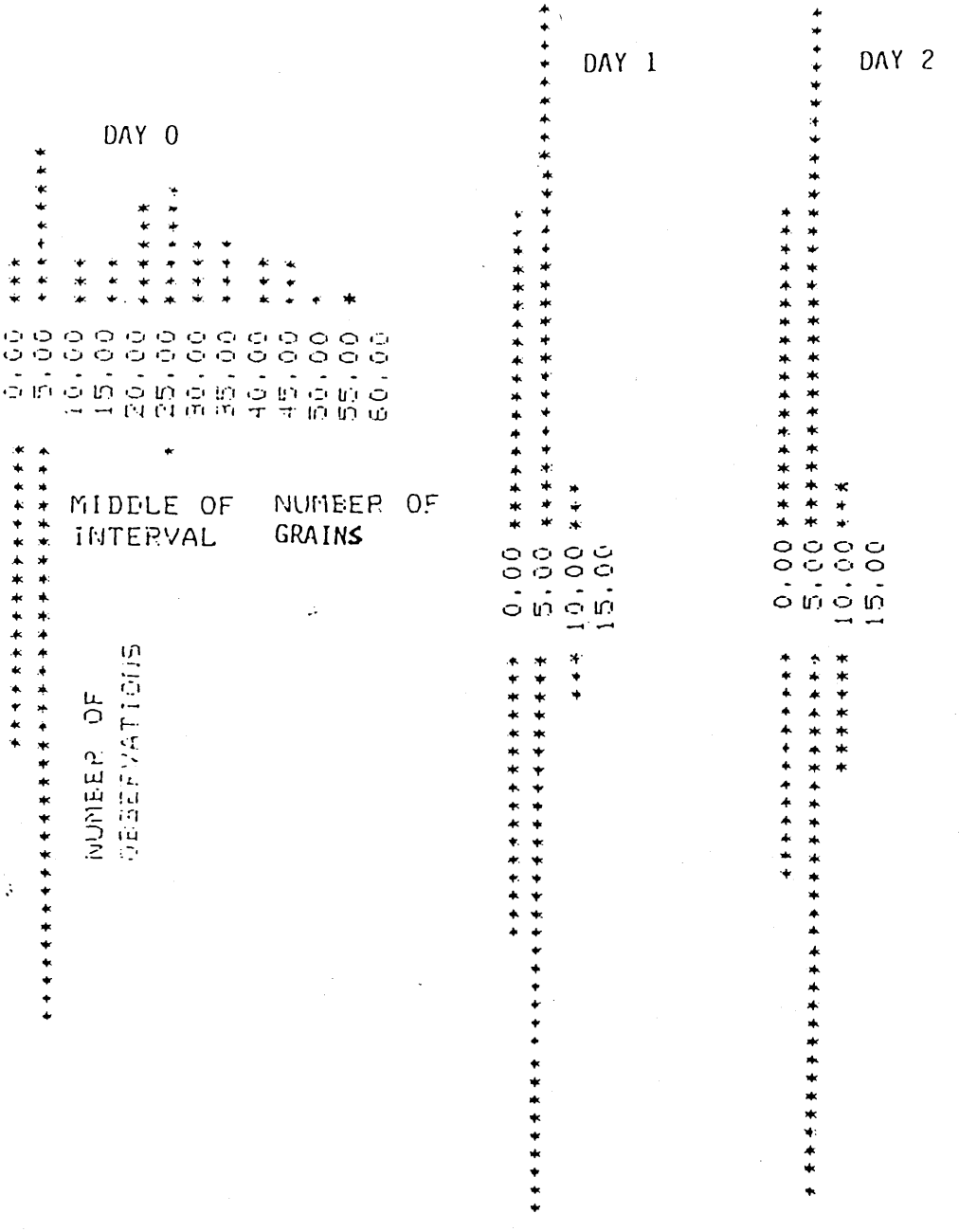
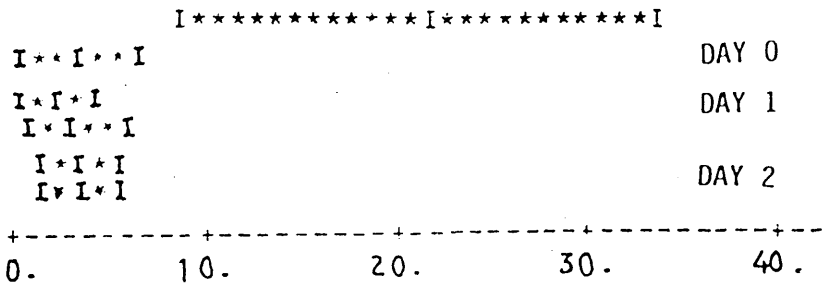


FIGURE 10(b): GRAPH OF 95% CONFIDENCE INTERVAL OF MEANS OF CONTACTING AND ISOLATED TG2 CELLS WITH IN VITRO CULTURED THYMOCYTES

Legend as for Table 20.



Distribution of grains over recipient cells in contact and isolated recipients after coculture with thymocytes cultured in vitro for 0, 1 or 2 days.

were observed in the neonate or adult strains tested. The observations of MEC^+ thymocytes in 3 to 6 week old mice in many strains and the occasional MEC^+ cell in neonates with the absence of detectable MEC^+ thymocytes in mature mice may reflect a transition from MEC^- to MEC^+ in neonates or a transition from MEC^+ in weanling to MEC^- in adults could not be determined by these experiments.

3:4.7 Metabolic co-operation between TG2 cells and weanling thymocytes cultured in vitro for varying times

If the loss of the MEC^+ population of thymocytes is due to conversion (? differentiation) to MEC^- then the phenotype change may also occur in culture where it would be easier to analyse. The persistence of MEC^+ cells in vitro was examined and the results of one of this series of experiments are shown in Table 20 and Figure 10 (a) and (b).

From Table 20 it can be seen that the ability to exchange metabolites with TG2 cells in the presence of [^{3}H]-hypoxanthine is lost after 1 day in culture. The means and standard deviations of grain counts over isolated and in contact cells are indistinguishable, but the histograms of the two groups show that the distribution of grain counts is different; Figure 11. reflects the usefulness of the large sample size in reflecting the underlying distribution trends of grain counts over isolated TG2 cells in contact with thymocytes.

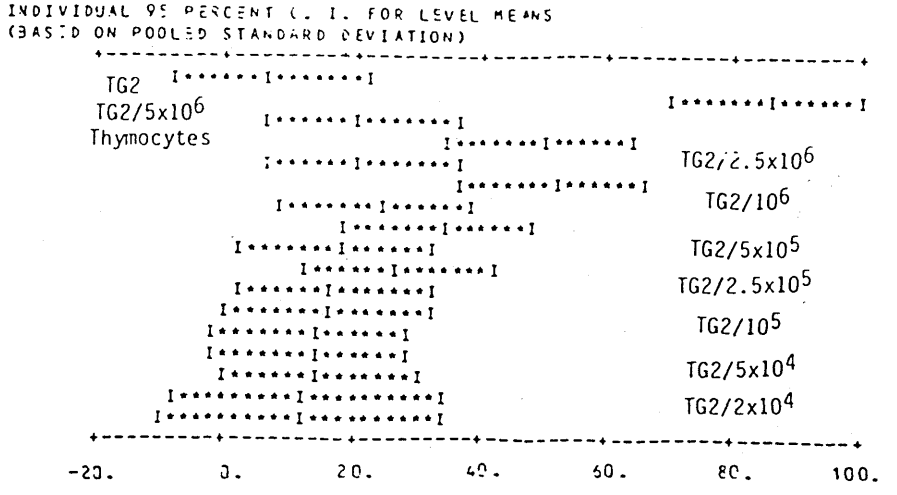
There was a considerable loss of viability after 1 day in culture which increases exponentially as the length of culture increases (data not shown). However, if non-viable cells were removed by centrifugation on Ficoll-Triosil (see Legend to Table 21) then the

TABLE 21: LIMITING DILUTION OF WEANLING CBA THYMOCYTES CO-CULTURED WITH TG2 CELLS IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

		Mean grain count/cell (SD)				
Co-culture	n	Cells in Contact	Isolated Cells	≈df	t	P
TG2	20	7.0(2.9)				
TG2/5x10^6 Thymocytes	20	56.95(16.9)	20.5(7.7)	24	7.23	0.00001
TG2/2.5x10^6 Thymocytes	20	49.3(17.6)	20.5(9.5)	29	4.47	0.00001
TG2/10^6 Thymocytes	20	51.5(17.9)	23.8(10.8)	23	5.7	0.00001
TG2/5x10^5 Thymocytes	20	33.2(20.0)	17.5(5.9)	27	3.2	0.00001
TG2/2.5x10^5 Thymocytes	20	26.8(15.3)	16.9(8.5)	26	3.9	0.00001
TG2/10^5 Thymocytes	20	16.0(9.3)	13.9(3.2)	37	2.198	0.0398
TG2/5x10^4 Thymocytes	20	13.8(4.4)	14.7(5.6)	38	-0.125	0.831(NS)
TG2/2x10^4 Thymocytes	10	12.6(2.3)	11.7(2.8)	18	0.7874	0.436(NS)

Thymocytes from sex matched weanling CBA siblings were dissociated enzymatically to a single cell suspension as previously described and added in known numbers to replicate cultures of TG2 cells (2x10^5/dish) grown overnight on 13mm glass coverslips in 50mm dishes in 5ml RPMI. The co-cultures were pulsed with 50µCi [^{3}H]-hypoxanthine for 3 hours at 37°C, washed with complete RPMI and fixed and processed for autoradiography as described before. Silver grains over 20 TG2 cells in contact with thymocytes and 20 isolated TG2 cells were counted, except in the cultures containing 2x10^4 thymocytes where 10 of each category were counted. The grain counts were subjected to two sample student's t test after means and standard deviations had been calculated.

FIGURE 11: 95% CONFIDENCE INTERVAL OF MEAN GRAIN COUNTS OF LIMITING DILUTION EXPERIMENTS



Legend as in Table 21
Distribution of grain counts over TG2 control and TG2 recipients in contact or isolated from decreasing number of thymocytes in the present of ^{3}H-hypoxanthine

loss of viability stabilised at 15% in subsequent days up to 4 days in culture.

The loss of demonstrable MEC^+ from thymocyte cultures could be due to MEC^+ cell death, conversion of MEC^+ to MEC^- cells, or the acquisition of a new specificity which does not permit gap junction formation between TG2 cells and a new MEC^+ phenotype. It is well documented that thymocytes can undergo differentiation and maturation in vitro, but without other means of discriminating MEC^+ from MEC^- thymocytes on some other phenotypic basis/bases all of the above events could be occurring.

From these experiments it is clear that the MEC^+ thymocyte is lost after only 24 hours in culture, but it is difficult to envisage ways of identifying the fate of the MEC^+ cells without additional knowledge about the MEC^+ thymocyte sub-population.

3:4.8 Analysis of MEC+ thymic lymphocyte frequency by limiting dilution in metabolic co-operation experiments

Experiments were designed to estimate the frequency of MEC^+ cells in the thymus utilising limiting dilution. The basic metabolic co-operation protocol using TG2 cells in the presence of $[^3H]$-hypoxanthine was performed, but to TG2 replicate cultures doubling dilutions of pooled sex-matched CBA thymocytes in single cell suspensions from $1x10^7$ to $2x10^4$ cells were added. The data from one of these experiments are shown in Figure 11 and Table 21.

From Table 21 and Figure 11, the mean grain count of TG2 cells in contact with thymocytes decreases as the number of added thymocytes decreases in the co-culture. This reflects the increasing number of TG2 cells in contact with thymocytes with grain counts similar to TG2 cells not in contact with thymocytes. This trend

is also reflected in the decreasing standard deviations of the sample means of TG2 cells in contact with thymocytes as thymocyte dilution increases. From the data of this experiment and other replicates (data not shown) the frequency of MEC^+ thymocytes in the weanling thymus is between 10^{-5} and $5x10^{-4}$.

However, this frequency is liable to be an underestimate as this technique only detects transfer and incorporation into the acid insoluble pool of recipient TG2 cell in contact with an MEC^+ thymocyte. As the technique excludes from the frequency estimates, all MEC^+ thymocytes not in contact with TG2 cells and MEC^+ cells which have formed gap junctions for period not long enough for the transferred label to enter the acid insoluble material in the TG2 cells. Although, at high thymocyte concentrations in the co-cultures, examples of positive transfer are easily detected, as the thymocyte concentration decreases so the incidence of positive transfer decreases. Extra replicate coverslips were scanned to detect all or any positive transfer between thymocytes and TG2 cells in the lower thymocyte concentrations of the limiting dilution experiments.

The defects in the protocol which lead to frequency underestimation in the limiting dilution experiments are lack of detection due to short co-culture period and lack of contact between MEC^+ thymocytes and TG2 cells. This could be reduced by increasing the co-culture period and increasing the TG2 recipient cell concentration, but both options have inherent drawbacks.

Increasing co-culture times has two disadvantages, one being phagocytosis of thymocytes by the TG2 fibroblasts as was observed in experiments using PBL/fibroblast co-cultures leading to cell loss and the possible acquisition of the wild-type HGPRT gene and gene products by the mutant TG2 cells. The other drawback, observed

in earlier experiments, was that a small number of isolated TG2 cells had high grain counts similar to TG2 cells in contact with thymocytes which were deemed to be MEC^+. This phenomenon could be due to either the loss of the MEC^+ thymocyte during fixation and autoradiography or the MEC^+ thymocyte had formed gap junctions with the TG2 cell, transferred label, detached and attached itself to another TG2 cell and repeated the process. The second option would in long co-culture produce progressively higher and higher background counts in the isolated recipient sample making statistical discrimination more difficult.

Increasing the TG2 cell concentration results in less and less TG2 cells being isolated. The formation of gap junctions between TG2 cells would mean that MEC^+ thymocytes in contact with contiguous TG2 cells would transfer the label to the recipient cell which could transfer to all other TG2 cells which were joined by gap junctions leading to a dilution of the TG2 incorporation/cell and an underestimation of the frequency of MEC^+ thymocytes. An increase in autoradiographic exposure time and an increase in sample size of both isolated and TG2 cells in contact would remedy this problem, but would increase the frequency of MEC^- classified as MEC^+ by binding to TG2 cells labelled by true MEC^+ thymocytes.

The above difficulties would make the interpretation and analysis of grain count data much more difficult and a more productive strategy of determining the surface antigen phenotype of MEC^+ thymocytes was decided upon. Once this was determined then the problems of frequency estimation, cell differentiation or MEC^+ phenotype loss in vitro could perhaps be resolved.

TABLE 22: METABOLIC CO-OPERATION BETWEEN TG2 CELLS AND PNA AGGLUTINATED AND NON-AGGLUTINATED WEANLING THYMOCYTES IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

Grain Counts over TG2 cells (mean (SD)) n = 50

Co-culture	In Contact	Not in Contact	≈df	t	P
TG2/PNA Agg Thytes	21.88(13.2)	12.14(5.16)	63	4.874	0.00001
TG2/PNA Non-Agg Thytes	12.2(4.2)	11.56(4.18)	96	0.875	0.39(NS)

Single cell suspensions were produced as previously described and incubated at $1x10^8$ cells/ml with PNA (Miles Ltd) at 1mg/ml for 30 minutes at room temperature. The incubated thymocytes were then overlayered on 5ml of foetal calf serum (FCS) in a 15ml sterile siliconised conical glass tube and left to sediment for 20 minutes at room temperature under 1g. After sedimentation, the unsedimented cells were removed from the FCS interface and the agglutinated cells removed from the bottom of the tube. Both groups of cells were washed once with SF RPMI and incubated with 0.2M D-Galactose SF RPMI at 37°C for 20 minutes. Incubation at 37°C was repeated after washing in SFRPMI with 0.2M D-Galactose for 5 minutes and the cells were then washed twice in D-Galactose RPMI and two changes of complete RPMI. The cell concentrations were readjusted to $1x10^7$ cells/ml and cocultured as before with TG2 cells ($2x10^5$) on glass coverslips at 37°C in the presence of [^{3}H]-hypoxanthine (10μCi/ml) in 5ml RPMI in 5cm tissue culture dishes. The cells were processed as before for autoradiography and the silver grains over 50 TG2 cells in contact with thymocytes and 50 TG2 cells not in contact with thymocytes were counted. Two-sample t tests were performed on the calculated means and standard deviations of the populations.

3:4.9 MEC^+ thymocyte surface phenotype using Peanut Agglutinin (PNA)

Initially, the lectin peanut agglutinin (PNA) was used to examine surface antigen phenotype of MEC^+ thymocytes. PNA binds to βD-Galactose residues and will agglutinate cells which bear unsialated carbohydrate moieties on their cell surface. In the thymus this lectin will agglutinate a large proportion of cells, and it is thought that this phenomenon is restricted to cortical cells reflecting their immaturity in differentiation. The protocol which was devised was to incubate single thymocyte suspensions ($1x10^8$ cells/ml) with PNA at 1mg/ml for 30 minutes at room temperature. The cells were then overlaid onto cushions of foetal calf serum and the mixture left to sediment at room temperature for 20 minutes. During this time the agglutinated cells settle to the bottom of the tube and the single cells remain at the FCS interface. Both agglutinated and non-agglutinated cells were then harvested and incubated with 0.2M D-Galactose in SF RPMI at 37°C for 20 minutes; the cells were then washed in serum free medium and incubated with 0.2M D-Galactose SF RPMI for 5 minutes at 37°C. They were then washed twice in D-Galactose medium and twice in complete medium. The cell concentration were adjusted to $1x10^7$/ml and the PNA separated populations were used in metabolite exchange experiments with TG2 cells in the presence of [^{3}H]-hypoxanthine. The data from one such experiment are shown in Table 22.

In Table 22 and replicate experiments, it was observed that PNA^-(12.7%) contained no detectable MEC^+ cells and the frequency of MEC^+ cells in the PNA^+ was slightly increased, but as before, the MEC^+ cells made up a minor fraction (less than 15%). From the published literature it is thought that the PNA^- thymic population

TABLE 23(a): TITRATION OF GUINEA PIG COMPLEMENT TO THYMOCYTES

Mean non-viable thymocytes (SD) 2 replicates 15 fields

Dilution	Mean (SD) NV	Dilution	Mean (SD) NV
Neat	62.7 (7.2)	1/32	38.1 (11.7)
1/2	56.1 (10.8)	1/64	16.2 (8.5)
1/4	55.8 (12.3)	1/128	10.7 (9.3)
1/8	58.8 (13.4)	1/256	8.78 (10.3)
1/16	49.9 (15.2)	1/512	9.29 (9.7)
Control (No Complement)	6.19 (6.13)		

A single cell suspension of $5x10^6$ thymocytes were incubated with agarose absorbed guinea-pig serum at 37°C for 40 minutes in complete RPMI 1640 containing 10% heat-inactivated foetal calf serum in duplicate. After incubation the cells were incubated in fluorescein di-acetate in serum-free (SF) RPMI 1640 (5µg/ml final concentration) for 15 minutes at room temperature. Fifteen fields were counted for viability using a fluorescence microscope with a x40 phase contrast objective.

TABLE 23(b): TITRATION OF MONOCLONAL ANTIBODIES AGAINST THYMOCYTES IN THE PRESENCE OF GUINEA-PIG COMPLEMENT

Mean (SD) non-viable cells 2 replicates 15 fields

Dilution / Antibody	Anti-Thy 1.2	Anti-H-2K^k	Anti-I^k	Anti-Lyt 1	Anti-Lyt 2
Neat	NT	NT	NT	65.37(12.1)	62.3(11.8)
1/2	NT	NT	NT	77.4(10.9)	27.2(8.4)
1/4	NT	NT	NT	61.5(13.2)	11.65(8.1)
1/8	NT	NT	NT	40.79(8.3)	9.7(8.9)
1/16	NT	NT	NT	26.06(12.3)	NT
10^{-2}	NT	44.3(6.1)	NT	NT	NT
10^{-3}	76.5(3.4)	44.9(7.3)	33.77(6.1)	NT	NT
$2x10^{-3}$	63.74(2.1)	37.5(5.9)	38.2(7.9)	NT	NT
$4x10^{-3}$	58.89(7.91)	37.3(4.8)	37.79(5.7)	NT	NT
$8x10^{-3}$	73.5(3.8)	42.78(7.6)	26.06(6.9)	NT	NT
$16x10^{-3}$	50.8(6.3)	39.6(6.4)	17.85(9.1)	NT	NT
$32x10^{-3}$	29.8(6.7)	32.6(7.9)	9.37(8.6)	NT	NT
$64x10^{-3}$	11.8(7.2)	22.9(8.4)	NT	NT	NT
$128x10^{-3}$	NT	22.79(4.7)	NT	NT	NT
Control (-C')	5.7(4.3)	7.3(3.2)	6.4(4.7)	7.2(5.7)	6.6(6.5)

Legend as for Table 23)a)

is mature, cortisone resistant and possess a surface antigen phenotype and reactivity similar to peripheral T lymphocytes (see Introduction). Thus, the surface antigen phenotyping was pursued with characterised monoclonal antibodies against surface antigens exhibited by thymocytes.

3:4.10 Analysis of MEC+ thymocyte surface antigens using monoclonal antibodies in complement mediated cytotoxicity (CMC)

To further characterise the surface antigen phenotype of this MEC^+ thymocyte minority, monoclonal antibodies against murine cell surface antigens, Thy 1.2, H-$2K^k$, IA^k, Lyt-1 and Lyt-2 (kind gifts of the Salk Cell Distribution Centre) were employed in complement mediated cytotoxicity (CMC) depletions and the resultant depleted populations used in the metabolite exchange protocol with TG2 cells in the presence of [^{3}H]-hypoxanthine. Initial titrations of complement and monoclonal antibodies were performed to determine optimum concentrations for maximum specific killing with minimum non-specific lysis. Fluorescein di-acetate was used to indicate viability by intracellular conversion of fluorescein di-acetate to trapped fluorescein in viable cells after treatment and the results of the titrations are shown in Table 23.

Monoclonal antibodies F7D5 anti Thy-1.2 mouse IgM, ascites fluid; 11-4.1 anti H-$2K^k$ mouse IgG_{2a}, ascites fluid; 10-2.16 anti I-A^k IgG_{2b}, ascites fluid; 53-7.313 anti Lyt-1 rat IgG_{2a}, supernatant; 53-6.72 anti Lyt-2 rat IgG_{2a} supernatant and $5x10^6$/ml cells were incubated with antibody for 20 minutes at room temperature, then incubated with guinea pig serum (final concentration 1/100) at 37°C for 40 minutes. The cells were then incubated for 15 minutes at room temperature with fluorescein di-acetate (5 µg/ml). An aliquot was

TABLE 24: METABOLIC CO-OPERATION OF CMC DEPLETED THYMOCYTES AND TG2 CELLS IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

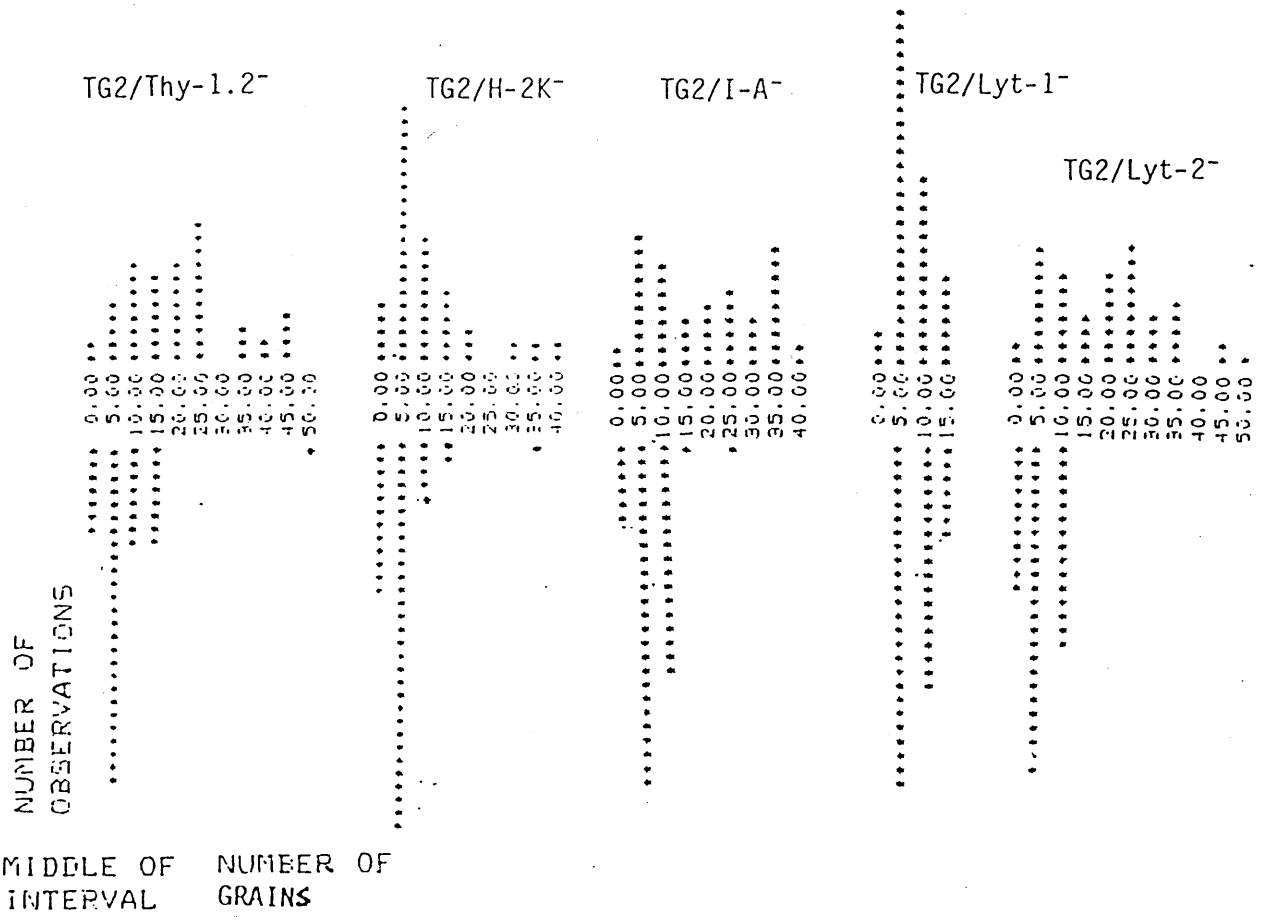
Mean grain counts over recipients (SD)

Co-culture	Total (%)	Cells in Contact	Not in Contact	≈df	t	P
TG2/Thy-1.2$^-$	(35)	20.2(11.9)	7.4(7.2)	80	6.51	0.0001
TG2/H-2K$^-$	(52)	11.1(10.4)	5.4(5.3)	73	3.43	0.001
TG2/I-A$^-$	(79)	19.1(12.3)	6.4(3.7)	57	7.06	0.0001
TG2/Lyt-1$^-$	(36)	7.3(3.9)	7.6(3.8)	97	0.47	0.64(NS)
TG2/Lyt-2$^-$	(43)	19.6(12.5)	5.2(2.9)	54	7.97	0.0001

CMC were performed on weanling CBA thymocytes as described in the legend to Table 23 and viable cells were recovered by centrifugation at 500g for 5 minutes over Ficoll (1.077g/ml). Viability was estimated as previously described, using fluorescein di-acetate and the cell concentrations returned to $1x10^6$ cells/ml. TG2 cells, cultured overnight at $2x10^5$ cells/5cm tissue culture plate on glass coverslips, were co-cultured with $1x10^6$ viable CMC depleted thymocytes in complete RPMI (5ml) containing 10μCi/ml of [^{3}H]-hypoxanthine for 3 hours. After co-culture the coverslips were fixed and processed for autoradiography as previously described. Silver grains were counted over 50 TG2 cells in contact with thymocytes and 50 isolated TG2 cells not in contact with thymocytes. The means and standard deviations were calculated for all samples and contacting and non-contacting samples subjected to two sample students t test for equality of means.

FIGURE 12: HISTOGRAMS OF GRAIN COUNT DATA FROM CMC METABOLIC CO-OPERATION

Legend as for Table 24.



removed from both replicates, pooled and 15 fields were counted under a fluorescence microscope for viability.

From the data presented in Table 23, it can be seen that ascites fluids prepared in mice of mouse/mouse monoclonal antibodies against Thy 1.2, H-2K^k and I-A^k epitopes produce optimum lysis at very high dilution, but the rat/mouse hybridomas against Lyt-1 and Lyt-2 antigens have lower titres, the antibodies being derived from tissue culture supernatants.

Using appropriate antibody and complement concentrations pooled weanling sibling CBA thymocyte single cell suspensions were subjected to large scale CMC with the five available monoclonal antibodies. Viable cells were recovered after washing by centrifugation on 1.077 (g/ml) Ficoll cushions, washing and testing for viability using fluorescein di-acetate. The cell concentration was adjusted to 1x10^6 cells/ml and tested for metabolic co-operation with TG2 cells in the presence of [^{3}H]-hypoxanthine. The data from one representative experiment from this series is shown in Table 24 and Figure 12.

CMC were performed on weanling CBA thymocytes as described in the legend to Table 23 and viable cells were recovered by centrifugation at 500g for 5 minutes over Ficoll (1.077g/ml). Viability was estimated as previously described, using fluorescein di-acetate and the cell concentrations returned to 1x10^6 cells/ml. TG2 cells, cultured overnight at 2x10^5 cells/5cm tissue culture plate on glass coverslips, were cocultured with 1x10^6 viable CMC depleted thymocytes in complete RPMI (5ml) containing 10µCi/ml of [^{3}H]-hypoxanthine for 3 hours. After co-culture the coverslips were fixed and processed for autoradiography as previously described. Silver grains were counted over 50 TG2 cells in contact with

thymocytes and 50 isolated TG2 cells not in contact with thymocytes. The means and standard deviations were calculated for all samples and contacting and non-contacting samples subjected to two sample students t test for equality of means.

From the data presented, it can be seen that MEC^+ thymocytes are present in the Thy 1.2, H-2K, I-A and Lyt-2.2 depleted populations but not in the Lyt-1 depleted cells. It should however be noted that the levels of residual viable cells after CMC depletion, for both Lyt-1 and Lyt-2 monoclonal antibody supernatants, are twice the reported values for mice of this age (Rothenberg and Triglia, 1983) and reflect the very low potency of these supernatants. However, the fact that the low antibody titre of the anti-Lyt-1 supernatant can totally ablate the MEC^+ population suggests that the whole MEC^+ population bears the Lyt-1 epitope on its surface. Treatment with both the I-A and Lyt-2 antibodies increases the frequency of detection of MEC^+ thymocytes/TG2 positive contacts. The particular antibody dilutions shown in Table 23(b) were chosen specifically to avoid 'bystander lysis', a phenomenon common in CMC. This is thought to occur when an antibody bound to a cell surface epitope fixes complement in the process of generating the complement lesion , the presence of excess antibody and complement induces the formation of complement lesions in adjacent cells which do not bear the epitope the antibody is directed to. The titre of the antibodies against Thy1.2 and H-2K was deliberately selected that cells bearing these epitopes at low levels would survive rather than be lysed in the CMC. From the probability data in Table 24 it can be seen that there is a reduced probability in the likelihood of equality of means between the recipients in contact and isolated

FIGURE 13: FACS DISTRIBUTION OF FORWARD LIGHT SCATTER VERSUS FLUORESCENCE OF ANTIBODY LABELLED THYMOCYTES

$1x10^4$ events were analysed to produce the distribution pattern of FLS vs fluorescence. The FACS II was gated to exclude dead cells and debris, and an arbitrary line drawn on the distribution to produce two populations of large cells which differed in fluorescence intensity. This arbitrary line was taken as the gating point betwen high Thy1.2 and low Thy-1.2 cell epitope density. The same first antibody titres were used in FACS experiments as was used in CMC experiments, in the case of this example $1/20x10^3$ anti Thy-1.2.

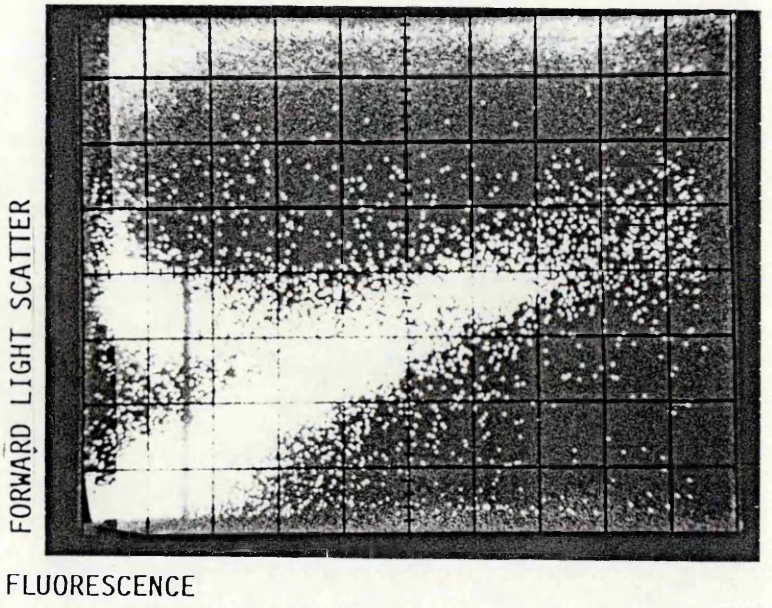
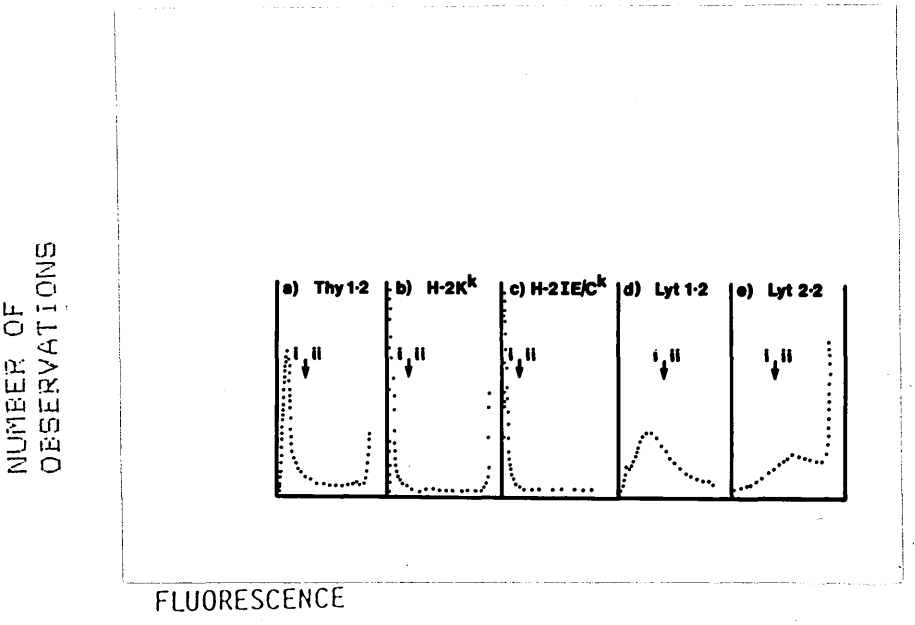


FIGURE 14: FACS distribution of fluorescence versus cell number of antibody labelled thymocytes.



After an arbitrary division of the treated cells, as described in the legend to Figure 13, $1x10^5$ events were analysed and sorted into the minor population. Plus and minus fractions were collected in 1ml of heat-inactivated foetal calf serum and the separated fractions wash thrice in 5ml of complete RPMI with 10% heat-inactivated FCS at 37°C. The black vertical bar indicates the division between high and low fluorescence, and percentage positive cells was 37.63 for Thy-1.2, 35.88 for H-2K, 21.98 for I-A, 43.25 for Lyt-1 and 78.3 for Lyt-2. 20µl of the specific fluorescein (Miles-Yeda) conjugated anti immunoglobulin was added to 100µl of cells ($1x10^6$/ml) in the specific anti-epitope antibody, except for Thy-1.2 (see legend to Figure 16) at the dilution previously described.

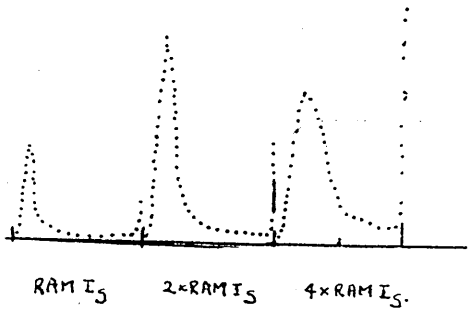
recipients in the anti-H-2K antibody treated cells, reflecting in the proportion of contacting thymocytes which were MEC^- as seen from Figure 12. This was also true for the anti-Thy-1.2 antibody treated population but to a lesser extent.

3:4.11 FACS analysis of MEC^+ thymocytes

In order to confirm the data generated from the CMC experiments it was decided that weanling CBA mouse thymocytes would be subjected to fluorescence activated cell sorting (FACS) to examine the behaviour of both surface epitope positive and negative cells with respect to their abilities to undergo metabolic co-operation with TG2 cells in the presence of $[^3H]$-hypoxanthine.

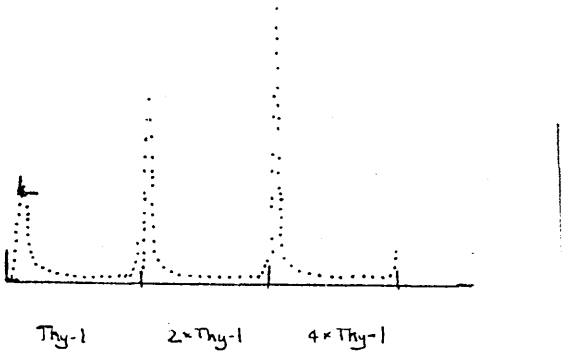
Thymocytes were prepared as before, but exposed to the epitope specific antibody at 4°C, before the addition of a second stage anti-immunoglobulin antibody for indirect immuno-fluorescence (IIF). The cells were held at 4°C for as short a time as possible before FACS sorting and the second antibody was added and mixed prior to insertion in the machine. The FACS was gated to exclude dead cells and debris from the analysis and $1x10^4$ events were analysed to generate distributions of forward light scattering (FLS) and fluorescence and histograms of fluorescence against cell number. From the FLS vs fluorescence, an example of which is shown in Figure 13, an arbitrary value was chosen, shown by vertical line in the second interval, to divide the population into two groups, each containing large cells. The same procedure was carried out for all samples analysed by the FACS. The distributions of fluorescence vs cell number for all antisera are shown in Figure 14.

FIGURE 15(a): TITRATION OF SECOND ANTIBODY TO Thy-1 LABELLED THYMOCYTES



$1x10^7$ thymocytes/ml from weanling CBA mice were treated with anti-Thy-1.2 monoclonal antibody at $1/20x10^3$ dilution and held at 4°C. 100µl of Fluorescein conjugated rabbit anti mouse whole IgG (RAM IgG) was added to 100µl of antibody treated thymocytes and the concentration of the second antibody doubled for each successive point in the titration. $4x10^4$ double labelled cells for each RAM IgG concentration were then analysed in the FACS and the distribution of cell number and fluorescence recorded.

FIGURE 15(b): TITRATION OF ANTI-Thy-1 ANTIBODY AGAINST CONSTANT SECOND ANTIBODY



The process was repeated with the conjugated antibody concentration remaining at a 1/1 ratio of RAM IgG and the concentration of mouse anti-Thy-1.2 antibody increased by double at each titration point.

TABLE 25: METABOLIC CO-OPERATION BETWEEN TG2 CELLS AND FACS SEPARATED CBA WEANLING THYMOCYTES IN THE PRESENCE OF [^{3}H]-HYPOXANTHINE

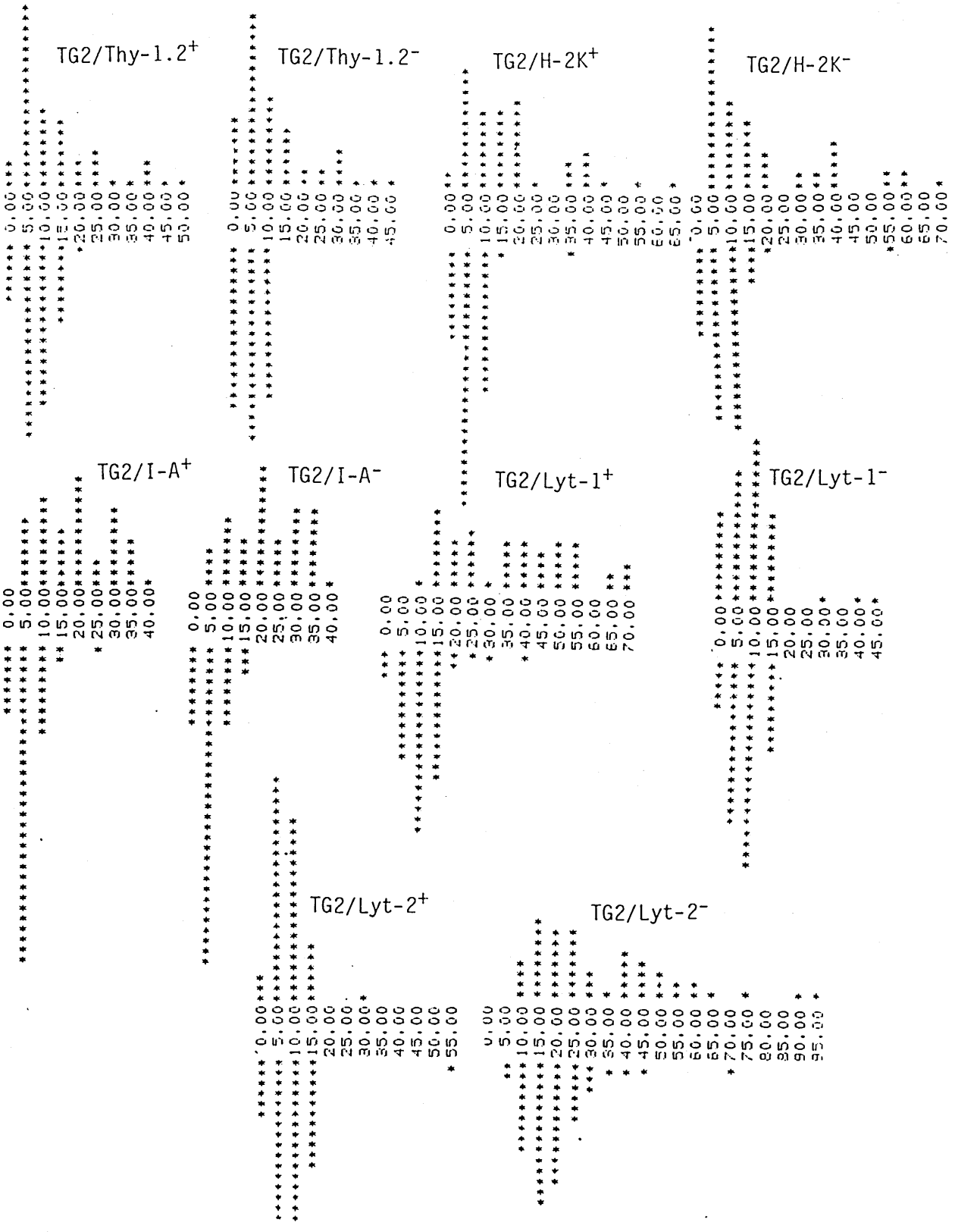
Grains counts over recipients mean (SD) n = 50

Co-culture	Arbitrary % Total	Cells in Contact	Not in Contact	≈df	t	P
TG2/Thy-1.2$^+$	37.63	14.18(12.7)	7.6(4.33)	60	3.474	0.001
TG2/Thy-1.2$^-$	62.37	11.86(11.3)	4.88(3.22)	56	4.198	0.0001
TG2/H-2K$^+$	35.88	17.94(14.6)	6.16(5.41)	62	5.338	0.00001
TG2/H-2K$^-$	64.12	19.3(18.0)	8.04(8.23)	68	4.032	0.0001
TG2/I-A$^+$	21.98	19.6(10.1)	6.08(4.09)	64	8.765	0.00001
TG2/I-A$^-$	78.02	21.54(9.7)	5.88(3.07)	58	10.88	0.00001
TG2/Lyt-1$^+$	43.25	37.72(17.3)	11.42(6.84)	63	9.595	0.00001
TG2/Lyt-1$^-$	56.75	9.54(8.42)	8.52(4.05)	70	0.772	0.443(NS)
TG2/Lyt-2$^+$	78.3	8.3(5.08)	9.52(7.86)	83	0.922	0.36(NS)
TG2/Lyt-2$^-$	21.7	33.5(20.4)	19.48(10.6)	73	4.312	0.00001

FACS analysis and sorting was carried out as previously described (see legend to Figure 15) with the thrice washed sorted cells ($1x10^5$ cells/well) co-cultured with $1x10^4$ TG2 cells per well grown on glass coverslips in 6x4 well plates in 1ml of heat-inactivated FCS complete RPMI containing 10μCi/ml of [^{3}H]-hypoxanthine and incubated at 37°C for 3 hours. After co-culture the cells were processed as previously described, with 50 contacting and 50 non-contacting TG2 cells counted.

FIGURE 16: HISTOGRAMS OF GRAIN COUNT DATA FROM FACS METABOLIC CO-OPERATION EXPERIMENT

Legend as for Table 25.



However, one difficulty arose in the IIF FACS analysis, the supplier of the fluorescein conjugated rabbit anti-mouse IgM antibody failed to produce the antibody as contracted, but by using a 5-fold increase in the concentration of the rabbit anti mouse IgG (whole molecule) with a fluorescein:protein ratio of 4.5, a fluorescent distribution of the anti-Thy1.2 antibody treated cells could be obtained and a titration of the primary and secondary antibody FACS fluorescent profiles is shown in Figure 15.

From Figure 15 it can be seen that by increasing the second antibody concentration for Thy-1.2 to five fold in comparison with specific primary and secondary antibodies the FACS can detect fluorescence in the Thy-1.2 antibody treated samples.

FACS sorted populations were subjected to co-culture with TG2 cells in the presence of [^{3}H]-hypoxanthine to compare the FACS treated samples with CMC depleted metabolic co-operation results. The data from one such experiment are shown in Table 25 and Figure 16.

The data presented in Table 25 and Figure 16 are consistent with results obtained in two previous FACS experiments except that the population sorted for I-A antigen. In all previous and two subsequent experiments the I-A negative population contained the MEC^+ population (data not shown). However, in this experiment using 3½ week old CBA female siblings, the arbitrary division with respect to fluorescence intensity splits the MEC^+ population into two parts, with the majority of the MEC^+ cells in the $I\text{-}A^-$ fraction. The FACS experiment was repeated twice subsequently, but this result could not be reproduced, although the arbitrary division between $I\text{-}A^+$ and $I\text{-}A^-$ was co-incidentally set at the same level by FLS as the CMC experiments $I\text{-}A^+$ lysis. From the FACS experiments the MEC^+ population surface antigen phenotype is in support of the CMC analysis

of PNA^+, Lyt-1^+, Lyt-2^- and I-A^-. However, from the FACS and CMC analyses it is clear that MEC^+ cells are present in both Thy-1.2^+ and Thy-1.2^- and in H-$2K^+$ and H-$2K^-$ populations. It should be noted that the FACS separated populations are in fact continua and the arbitrary division of fluorescence intensity splits this continuum into high versus low and zero surface epitope density. There also exists a difference in sensitivity between FACS and CMC epitope detection. The FACS can detect epitope densities above $3x10^3$ molecules/cell whereas CMC will produce cell lysis at a minimum epitope density of 10-100 molecules/cell in saturating conditions (J. Fothergill, personal communication).

3:5 Double CMC using anti Thy-1.2 and anti H-$2K^k$ Monoclonal Antibodies against MEC^+ Thymocytes

The CMC experiments were deliberately set up at low cell density to eliminate 'bystander lysis', a phenomenon encountered when performing CMC at high cell density with excess complement present. The result of this decision was that in CMC experiments, the antibody concentration chosen did not lyse every cell bearing the specific epitope and only cells that were negative or expressed low levels of the epitope survived the treatment. The decision to lyse only high and medium epitope density bearing cells was that for Thy-1.2 and H-2K, MEC^+ were detected in the residual populations after CMC. This result is also reflected in the presence of MEC^+ cells in both Thy-1.2^- and H-$2K^-$ FACS sorted populations as a result. Thymocytes were subjected to double CMC selection for Thy-1.2 and H-2K antigens. These experiments were performed using the same CMC protocol twice on the one population except that after the first CMC the cells were washed twice and the CMC performed again. After the second

TABLE 26: DOUBLE COMPLEMENT MEDIATED CYTOTOXICITY FOR Thy-1.2 AND H-2K^k THYMOCYTE SURFACE ANTIGENS USED IN METABOLIC CO-OPERATION WITH TG2 CELLS IN THE PRESENCE OF [^{3}H]HYPOXANTHINE

Grain counts over Recipients Mean (SD) n = 50

Co-Culture	Non-Viability (%)	Cells in Contact	Not in Contact	≈df	t	P
TG2/Thy-1.2$^-$	87.13(7.7)	8.66(6.5)	7.56(4.78)	93	0.735	0.47
TG2/H-2K$^-$	68.1(8.9)	7.96(3.95)	8.22(3.8)	97	0.365	0.631

The CMC was performed as previously described (See Tables 24 and 25) except after the first CMC the cells were washed twice in SF-RPMI at room temperature and subjected to identical CMC. After the second CMC the viable cells were recovered after washing with heat inactivated complete RPMI by centrifugation on 1.077g/ml Ficoll, washed and the cell suspension readjusted to $1x10^7$ cells/ml. $1x10^7$ cells were then co-cultured with TG2 cells ($2x10^5$ cells/5cm dish) established overnight on glass coverslips for 3 hours in the presence of [^{3}H]-hypoxanthine (10μCi/ml) in 5ml RPMI (complete heat inactivated). The coverslips were then fixed and processed as previously described.

CMC viable cells were recovered on a 1.077g/ml Ficoll gradient and used in metabolic co-operation experiments as before. The data from one such experiment are shown in Table 26.

From Table 26 it can be seen that MEC^+ cells bear Thy-1.2 and H-2K epitopes on their surfaces, as double CMC abrogates metabolic co-operation; however, in the Thy-1.2^- population one lymphocyte in contact with a TG2 cell did appear to be able to communicate by gap junctions as the TG2 cell had 37 grains over it, suggesting that this MEC^+ lymphocyte was Thy-1.2^-.

From the accumulated data the phenotype of MEC^+ thymocytes for the five surface antigens tested can be summarised as PNA^+, Thy-1.2 high and low, H-2K high and low, Lyt-1^+, I-A^- and Lyt-2^-. This phenotype would place MEC^+ thymocytes in the helper cell lineage with respect to Lyt antigen phenotype, but the presence of low Thy and high H2-K suggest maturity, whereas the high Thy low H-2K suggests an immature thymic lymphocyte differentiation stage, as does the PNA^+ phenotype.

CHAPTER 4

DISCUSSION

'Non ridere, non ligere, neque detesare, sed intellegere'

('Not to laugh, not to lament or to curse, but to understand')

Spinoza

RAJI cells are classified as pre-B cells by their lack of membrane bound immunoglobulin (mIg) and presence of heavy and light immunoglobulin chains in their cytoplasm. However sIg can not be induced by all the conventional inducing agents (Paige, 1978), they express C3b receptor, lack Fc receptor, but they also possess Ia antigen (Preudhomme , 1978) and B cell differentiation antigen (Basch et al, 1978). From analysis of RAJI karyotype the original cell has a translocation in chromosome 14 to chromosome 8 and the adherent subline has t (1:Y) (Nyormoi et al, 1973) translocation also. Although RAJI cells contain integrated Epstein-Barr virus (Klein, 1975) they do not produce mature virus particles and only produce early antigens when treated with tetradecanoyl phorbol acetate (TPA), which induces virus capsid antigen in virus producing lines (Yamamoto and Bauer, 1981).

Early pre-B cells have been shown to produce only μ heavy chain but no light chain in their cytoplasm (Burrows et al, 1979) whereas RAJI produces both K light chain and μ heavy chain. In later experiments where cytoplasmic μ chain were immunoprecipitated it was found that two species of μ heavy chains could be detected corresponding to membrane and secreted forms (Cushley, personal communication), with the conclusion being reached that a defect in

glycosylation inhibited Ig production, as mouse pre-B cells synthesize and secrete μ heavy chains but not light chains (Levitt and Cooper, 1980).

From the surface antigen data and presence of both cytoplasmic secreted and membrane forms of immunoglobulin it seeems most likely that RAJI is not a pre-B cell, but a mature IgM B cell or a memory IgM B cell transformed by EBV.

In co-cultures of RAJI with PBL, the observed hypoxanthine incorporation exceeds the expected incorporation for half quantities of each cell type. This phenomenon could be accounted for by metabolic co-operation between the wild-type PBL and HGPRT$^-$ RAJI cells, but the increase in grain count over RAJI cells in contact with PBL is only approximately 15% greater than RAJI cells not in contact with PBL and is not statistically significant. There is also a slight increase in the incorporation of RAJI cells alone and isolated RAJI cells in the co-culture of about 4%.

A possible reason for this disparity is synergism between RAJI and PBL in co-culture. In overnight co-cultures this phenomenon was more apparent and to test the dependence of the presence of RAJI cells varying quantities were added to PBL co-cultures. As can be seen in Figure 4 (Results section), the presence of RAJI cells in the co-cultures stimulates the uptake of label, but high levels of RAJI cells decrease the amount of label incorporated.

When pool sizes of acid soluble and acid insoluble material are considered all concentrations of RAJI cells decrease incorporation into the stable acid insoluble RNA and DNA, but increase incorporation into the acid soluble pool of nucleotides and small metabolites. High concentrations, ie 2:1 RAJI/PBL, the acid soluble pool size decreases but not to the level of PBL alone. As the amount of increase

in incorporation into RAJI cells as measured by autoradiography is not sufficient to account for this large increase, it must be due to increased label uptake by the PBL.

Many primary cells do not grow well in culture and their survival can be enhanced by the addition of a 'feeder' layer. In stromal cells much of this synergism is accounted for by the formation of gap junctions between primary cells and 'feeders' resulting in the coupling of intracellular metabolisms. However growth enhancement in co-culture has been demonstrated with cells that do not form junctions, notably L929, and by cell 'conditioned' medium. Two reasons have been advanced for co-culture and 'conditioned' medium growth enhancement of primary cell lines, leakage by the 'feeder' of intermediate metabolites essential for primary cell growth and the production of growth stimulating factor(s) by the feeders.

It has been demonstrated that PBL, unless stimulated with mitogens, rapidly die in culture. If RAJI cells provide sufficient metabolic support by low level gap junction formation and 'leakage' of essential intermediate metabolites into the extracellular medium, the increased survival of PBL in cocultures would account for some of the increase in coculture incorporation. Moreover, it has been shown that PHA stimulated T cell cultures have a marked increase in purine nucleotide and adenine metabolism, with the greatest increase occurring in HGPRT function (Ravio and Hovi, 1978) as well as de novo nucleotide synthesis.

PBL are a mixture of T and B lymphocytes and although obtained from young apparently healthy donors, are dynamic reactive cells, as has been seen from the variations in incorporations within and between individual samples. If RAJI co-culture provides sufficient support for survival and growth of PBL it is not inconceivable that

normal T and B cell interactivities should not continue. Furthermore, if RAJI produce growth and/or activating factors, these would further increase incorporation in RAJI/PBL co-cultures. These plausible reasons do not include the interactions of PBL on RAJI cell metabolism and the consequent effects this may have on the PBL population or the role of RAJI cells as stimulators and activators of allogeneic responses of the PBL against allogeneic epitopes on the RAJI cells.

As mentioned in the Results section, PBL have high levels of intracellular material in their acid soluble pools, especially when labelled with [^{3}H]-hypoxanthine, which should make PBL good donors in nucleotide exchange or metabolic co-operation experiments. As a result the experiments of Oliviera-Castro and Hulser were repeated using nucleotide exchange and metabolic co-operation.

In Table 7 it can be seen that not even low level transfer was detected at any time point during PHA stimulation in metabolite exchange with 3T3 cells. In metabolic co-operation experiments with TG2 cells, theoretically a more sensitive technique (see Introduction, Figure 1), although the differences between TG2 cells in contact or not with PBL were marginally larger no statistical difference was observed. Once again several reasons can be postulated for this disparity of lack of coupling seen in metabolite exchange techniques and the reported electrophysiological data.

The most plausible reason is the differences in sensitivity of detection of two methods of measuring cell/cell coupling. Other possible reasons include, high specificity in gap junction formation in PBL as the electrophysiological experiments were performed between lymphoid cells and not between lymphoid and stromal cells as in these cases, or differences in PBL/stromal cell interaction. The result of which may be low rates and numbers of gap junctional channels

formed with high rates of turnover. This latter case would be detected by electrophysiology, but would be difficult to detect by metabolite exchange. In order to overcome the specificity problem, if it exists, it would be necessary to obtain stromal cells of the same haplotype as the lymphocytes under investigation and although most volunteer donors will permit repeated venepuncture for PBL, some resistance to tissue biopsies would be inevitable and not unreasonable.

To perform lymphocyte/lymphocyte co-cultures it would be necessary, initially, to find a substrate to which lymphoid cells would bind and remain adherent not only during co-culture, but for autoradiographic processing. One such substrate, collagen gels, has recently been described (Shields et al, 1984), but the major difficulty remains in adequately determining donor from recipient cells in the co-culture. Either donor or recipient marking would be sufficient, as discrimination is only required to ensure that grain counted cell pairs are donor to recipient and not donor/donor, which would be incorrectly attributed to metabolic co-operation. The substance used would have to be nondiffusible into the extracellular space, as diffusion and uptake would lead to mis-classification in donors or recipients, and must not be small enough to pass through gap junction channels to give unequivocal identification as either donor or recipient.

The confirmation of the comment of Smets et al (1980) that somatic cell hybrids between L929 and PBL, both MEC^- by metabolite exchange, can and do form demonstrable gap junctions, was predictable from the work of Arzania et al (1974) and Bols et al (1979) and McDonald (1982) utilising parental cells, $HGPRT^-$ MEC^+ human fibroblasts/C1-D1 (a TK^- derivative of L929); MEC^- chick embryo erythrocytes/A9 cells; and MEC^- $LMTK^-$ (a TK^- derivative of L929)/MEC^-R5/3 ($HGPRT^-$ derivative of embryonal carcinoma line PC13) respectively. In the

Arzania paper it was demonstrated that in MEC^+/MEC^- hybrids, the MEC^+ phenotype is dominant; the work by Bols also showed MEC^+ phenotype dominance and that the inactive erythrocyte mucleus would complement the MEC^- phenotype of A9 cells, but only after 4 days when the chick nucleus is reactivated. The work of McDonald has further strengthened the belief that genetic complementation can provide for the restoration of the MEC^+ phenotype. In the A9/PBL hybrids it was decided that selection of adhesion was a priority to enable metabolic co-operation testing, which demonstrated the MEC^+ phenotype of the PA hybrids.

Inability to demonstrate specific human chromosomes in mouse/human hybrids is well documented (Smets et al, 1979; Junker, 1982) due to the specific loss of human genetic material. The hybrids show communication comparable to any of the known MEC^+ lines and the decision not to initially clone individual hybrids at the outset after HAT selection was a fortunate one. By the time cloned cells had been grown up to provide stocks for storage in liquid nitrogen and cells for metabolite exchange testing, the genotypic reversal due to selective loss of human chromosomes would have been well advanced. The presence of the human isoenzyme for glucose-6-phosphate dehydrogenase and the presence of an active HGPRT gene product amply demonstrates the human X-chromosome contribution to the hybrids but the human X-chromosome could not be detected karyotypically. The loss of human chromosomes was also accompanied by a reversion to A9 morphology amongst some of the hybrids, but this did not affect their ability to form gap junctions. It was not possible to determine which of the human lymphocyte chromosomes was responsible for the MEC^+ phenotype or morphological phenotype conversion because of the rapid chromosome loss, as was commented on by Smets et al (1979).

From the various fixation agents tested on [^{3}H]-hypoxanthine pulsed cells it is obvious that while glutaraldehyde fixation does increase the grain counts in treated cells, it also causes the release of the acid soluble material from the cell into the extracellular medium, especially during long fixation times. This phenomenon is probably due to the cross linking of amino groups of glycoproteins in the cell bi-layer. While this is the case, it must also to some extent retain labelled material in the cytoplasm and nucleus from the increase in glutaraldehyde fixed autoradiographs. There are two possible reasons for this: either the glutaraldehyde fixation fixes the labelled material in situ, or inactivates the enzymes which would degrade stable RNA and DNA into acid soluble material.

As previously mentioned in the Introduction, lymphoid progenitors are impossible to identify as no reliable markers have been described and their existence is only demonstrated by their identifiable progeny in vitro. It is only recently that in vitro systems have been described which allow the in vitro production of B cells from their progenitors in bone marrow (See Introduction). The labelling of bone marrow cells and seeding them onto pre-existing bone marrow stroma to assess gap junction mediated coupling would be feasible, but the identification of the haemopoietic cell interacting with stromal cells would prove difficult due to homogeneity of morphology and lack of cell markers and functional assays. Likewise lymphoid/stromal interactions in immune responses would prove difficult as it is not known if there is specificity between reactive lymphoid cells and antigen presenting cells to the antigen eliciting the immune response. The only feasible way of performing metabolite exchange experiments in both bone marrow and immune response systems would

be to find a HGPRT$^-$ mouse strain (a Lesch-Nyhan mouse) and grow the stromal cells of marrow and lymph nodes with HGPRT$^+$ lymphoid cells in the presence of [^{3}H]-hypoxanthine and to date no such HGPRT mouse has been described.

Lymphoid development in the thymus is the most logical area to focus on as there is limited cell heterogeneity and well developed and described markers for different differentiation stages. It was extremely fortuitous that the ages of the mice supplied initially coincided with the period when the cellularity of the thymus is increasing rapidly. In initial experiments using weanling (3-4 wk.) mice high levels of metabolite exchange were found using the uridine nucleotide transfer technique.

It appeared that the cells capable of forming gap junctions were a significant proportion of cells that adhered to the 3T3 cells. However, from the histograms of grain density over recipients, it is clear that not all adherent cells were capable of forming junctions, especially in the high density fraction. It is impossible to say whether or not these cells would have formed gap junctions given sufficient co-culture time or whether they are effector cells reacting to 'foreign' epitopes on 3T3 cells.

The necessity for cell-cell contact to produce higher grain counts over recipient cells was demonstrated by the difference in grain counts in cultures where the thymocytes were physically separated from 3T3 recipients, in comparison to 3T3 cells in direct contact with identical prelabelled thymocytes. The gap junction mediated nature of the transfer was shown by the lack of difference in the grain counts over isolated and MEC$^-$ A9 cells in contact in comparison with MEC$^+$ TG2 recipients in the presence of [^{3}H]-hypoxanthine. Gap junction forming thymocytes were not the predominant type in the

mixture, with many of the cells in contact not transferring label and with a minor fraction of isolated recipients with grain counts similar to contacting positive cells.

Cell-cell adhesion is a prerequisite for gap junction formation, but not all thymocytes added to the 3T3 cultures adhered to the stromal cells and some recipient cells without thymocytes bound would seemed to have formed junctions transferred label and detached. It is impossible to say if these thymocyte/stromal cell interactions terminated prior to the end of co-culture or were simply lost during subsequent processing.

If the thymocyte/stromal cell interactions are considered with respect to time then any donor thymocyte which forms gap junctions with a 3T3 cell at the start of the co-culture would be expected to exchange a greater amount of label than interactions occurring later in the co-culture period. The above argument assumes that the net junctional channels per interaction are equal and that the anabolic processes in the donor thymocytes are also equal, ie as the length of co-culture increases, so more of the label in the thymocytes is converted to non-exchangeable stable RNA and DNA. However, there is no way that the metabolic states of the donor and recipient pair can be assessed at the end of the co-culture; all that remains on fixation is the net result of their interactions.

From the cell density it can be assumed that the larger donor thymocytes are metabolically more active, i.e at or nearing cell division, where greater demands will be placed on their nucleotide pools, whereas the smaller donor cells may be in G_0 or entering G_1, thus leaving more free label for exchange.

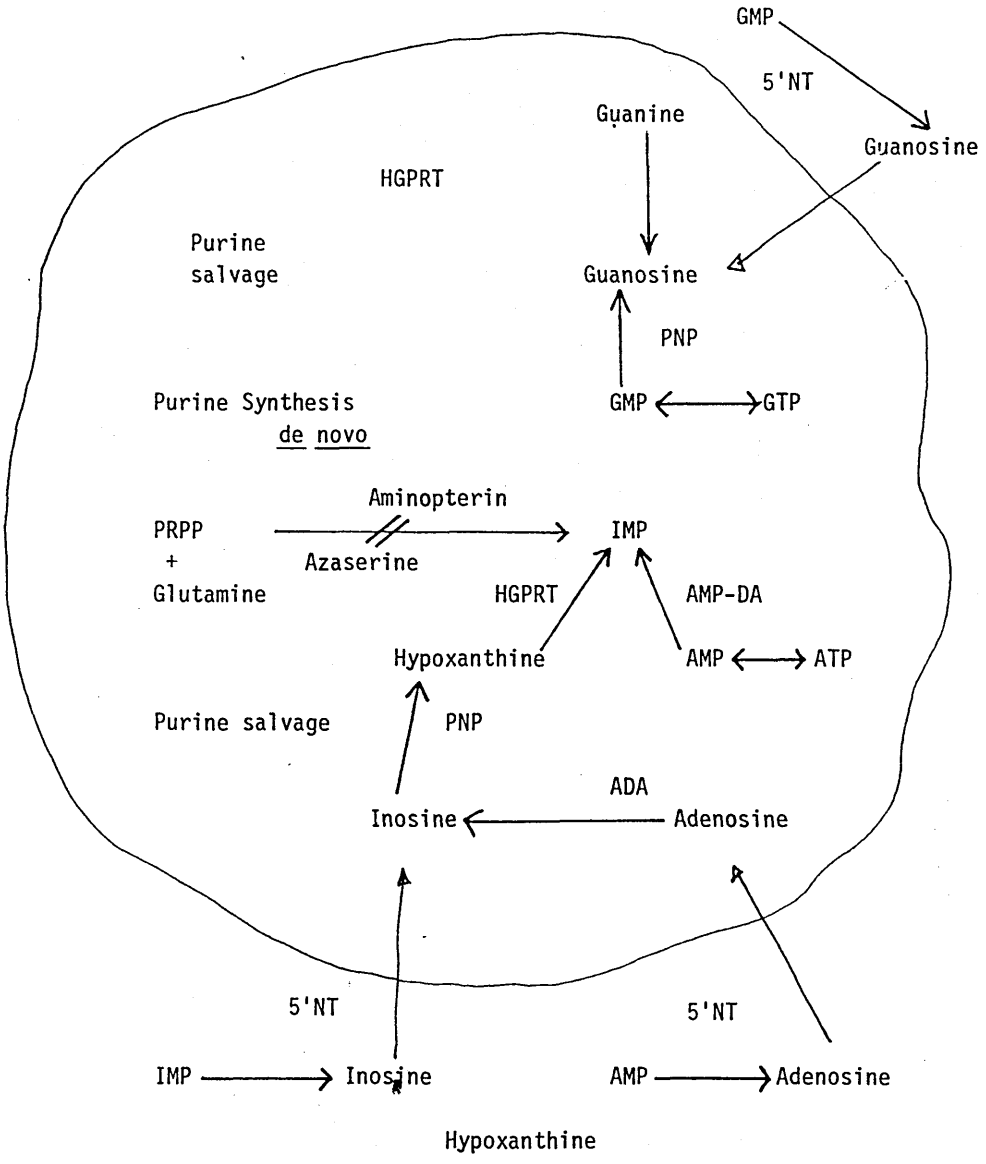
From the incorporation of thymidine, uridine and hypoxanthine by thymocytes, it is evident that thymic lymphocytes have substantial differences in their ability to incorporate labelled bases and nucleosides. Although the incorporation into acid soluble material is comparable for both purines and pyrimidines, the purine incorporation, as assessed by hypoxanthine, into acid insoluble material, is equal to the sum of the uridine and thymidine incorporation into the same pool. This observation is all the more remarkable as the specific activity of the labelled pyrimidines exceeds that of the hypoxanthine by 5-6 fold.

Increases in purine utilization has been reported for PHA stimulated T lymphocytes (Raivio and Hovi, 1978) and dividing B lymphoblastoid cells. This phenomenon has been ascribed to the catalytic activities of Ecto-5' nucleotidase (see Introduction) when de novo synthesis of purines is limited and/or exogenous purine nucleotides are available. However, in the thymus, cortical thymocytes show 4-5 fold lower 5' NT activity than medullary thymocytes, similar to PBL (Ma et al, 1982). However, there seems to be a high level of nucleoside re-utilization in the thymus as 63-67% of [^{3}H]-TdR incorporated by thymocytes into DNA is salvaged and re-utilized (Ma et al, 1982).

As the fate of most thymocytes is intrathymic death (McPhee et al, 1979) and on thymocyte death, a Zn^{++} dependant nuclease reduces thymocyte DNA to single nucleotides (M. Ulanovsky, personal communication) there must exist in the thymus, high extracellular concentrations of purine and pyrimidine nucleotides. However, given the incorporation of [^{3}H]-hypoxanthine into both acid soluble and insoluble pools, the purine anabolic pathways must be functioning normally in thymic lymphocytes. From PHA stimulated T cells, labelling

FIGURE 16

CELLULAR PURINE METABOLISM



Abbreviations

5'NT	: Ecto 5' Nucleotidase
ADA	: Adenosine deaminase
PNP	: Purine Nucleotide Phosphatase
HGPRT	: Hypoxanthine Guanine Phosphoribosyl Transferase
(d)(r)GTP	: Guanosine Tri-phosphate
dGTP	: Deoxyribosyl GTP
AMP	: Adenosine(d)(r)monophosphate
AMPDA	: Adenosine(d)(r)monophosphate deaminase

with ^{14}C-Hx resulted in adenine nucleotides being more heavily labelled than guanine nucleotides in the acid soluble pool but the nucleic acid fraction contain more guanine than adenine nucleotides (Raivio and Hovi, 1979).

The presence of high efficiency nucleotide, nucleoside and base scavenging systems has an obvious advantage to the cells involved. It requires much less energy to produce nucleotides by salvage than it does to synthesise nucleotides de novo. For active dividing cells, the ability to scavenge the nucleic acid precursors must have great advantages. However, for resting thymocytes, uptake of purine and pyrimidine precursors would have severe disadvantages given the nucleotide enzyme phenotypes of immature and mature thymocytes. As discussed in the Introduction (see section 1:10) immature cells exhibit the ADA^{high} PNP^{low} phenotype whereas mature cells have ADA^{low} PNP^{high} phenotype. From Figure 16 it can be seen that both these phenotypes will lead to accumulations of toxic levels of nucleotides (Ma et al, 1982).

The result of this enzyme phenotype is primarily by phosphoribosylpyrophosphate (PRPP) depletion leading to inhibition of endogenous pyrimidine synthesis (Ishii and Green, 1973) and that adenosine resistant cell lines show increased activity of ADA pathway (Fernandez-Mejia et al, 1984).

The importance of these enzymes in the generation of functional T cells has been demonstrated by the use of specific enzyme inhibitors, excess purine nucleosides and rare human mutants with disfunctions of these enzymes (see Introduction - 1:10).

As stated previously, formation of gap junctions requires close interactions and adhesion between the cells forming the junctions. CBA and C3H thymocytes adhere to xenogeneic fibroblasts (TG2 cells) of hamster origin, non syngeneic fibroblasts (BALB/c 3T3 cells) and sex different thymic stromal cells as well as to sex-matched syngeneic stromal cells. How is this interaction brought about? Are thymic lymphocytes merely 'sticky' or do they express receptor(s) for determinants on this range of cells? High levels of allo- and xeno-reactive lymphoid cells have been reported (Matzinger and Bevan, 1977; Finberg et al, 1978), but until much more is known about the surface receptors of thymocytes, it is impossible to say if thymocytes bound to stromal cells, be they autologous or heterologous, are binding to common structures or allotypic structures or both. The increased binding in heterotypic thymus combinations may reflect thymocytes detecting allotypic determinants and by the distribution of bound thymocytes it may be possible to define sub-types in both stromal and thymocyte populations.

Two types of rosetting stromal cells have been reported: an H-2 I-A/E$^-$ macrophage and an H-2 I-A/E$^+$ medullary dendritic cell and the dendritic cells can act as a presenting cell for exogenous antigen (Kyewski and Kaplan, 1982; Kyewski et al, 1984). Reticulum cells have been shown to express H-2 I-A and regulate mature medullary thymocyte proliferation by the secretion of IL-1 and PGE_2 (Papiernik and Homo-Delarche, 1983) and one group has found that dendritic, macrophage and thymic nurse cell associated thymocytes contain CTL precursors (Fink et al, 1984). Using the associations of thymocytes with these defined groups of stromal cells it may be possible to define differences in thymocyte types which bind to individual stromal cell type and discover the mechanisms functioning in these interactions and their outcome for the bound lymphocytes.

Jotereau et al (1980) reported cyclic waves of lymphoid influx into the developing avian thymus and this phenomenon may account for the incidence of peak MEC^+ thymocytes observed in weanling and infant mice. However, the MEC^+ peak may be due to the increasing cellularity of the thymus by proliferation of thymocytes which have already entered the organ placing these cells not as new migrants into the organ but differentiating inhabitants of the thymus. The paucity of MEC^+ cells in foetal and neonates and the apparent absence in juvenile and adult animals could reflect real fluctuations in the numbers of MEC^+ phenotype cells or perhaps at the times tested the MEC^+ content was tightly bound to the thymic stromal constituents.

The loss of MEC^+ phenotype *in vitro* could have been (as previously stated), preferential death of MEC^+ cells, increase in specificity of gap junction formation, decrease in rate of gap junction formation and cell differentiation from MEC^+ to MEC^- phenotype. All the above reasons also apply to the observations on MEC^+ phenotypes of mice of different ages. Removal of MEC^+ cells by Ficoll treatment is unlikely as no viable cells were seen in pellet on washing and fluorescein di-acetate treatment. If the specificity had increased or the rate of junction formation decreased, the assay system, the large sample size and the statistical analysis would have detected some residual albeit reduced gap junction formation, unless both these events happen simultaneously and to all members of the population.

Of the listed possibilities, this only leaves differentiation from MEC^+ to MEC^- phenotype or preferential MEC^+ cell death. *In vitro* thymocyte differentiation has been reported, as has the acquisition of mature T cell phenotypes *in vitro* (Ceredig et al, 1982; Rothenberg and Triglia, 1983), but until a MEC^+ surface marker appears, both these possibilities must exist.

What is the role of this MEC^+ phenotype? To answer this question, the function of both gap junctions in general and the thymus, must be considered. As stated in the Introduction (see Section 1:5), gap junction formation would appear to have many roles in the regulation and control of cell function. A whole range of functions, from compartmentation in early embryogenesis, through limb and tissue formation, have associated changes in gap junction formation. Gap junctions assist the transfer of action potentials in excitable cells, damp down sub-threshold stimuli, increase smooth muscle contraction, assist in the generation of synchronicity of germ cells, support developing oocytes and maintain oocyte meiotic arrest. Disfunction produces abnormality and dysplasia in developing embryos whereas function can confer a 'homozygous normal' phenotype on heterozygous mutant/normal individual and diminution of gap junction formation may contribute to neoplastic or malignant cell generation.

How do these myriad effects bear on the generation of T lymphocytes from committed precursors in the bone marrow? In two ways pre-T cells resemble developing embryonic cells, they are only partially differentiated and do not exhibit the phenotype of the T cells in surface antigens, function and location. They must pass through the periphery, perhaps under the influence of hormones secreted from the thymic epithelium and enter the thymus. However, there is some evidence that pre-T cells develop the ability to recognise self prior to entry into the thymus (Morrissey et al, 1982; Chervenak et al, 1983; Chervenak et al, 1985) and that thymocytes appear to ignore Class I MHC determinants (Von Boehmer and Schubiger, 1984) on epithelial cells of the thymus. Recent work suggests that the thymus dictates MHC specificity of T cells restricted to Class II MHC molecules (Kast et al, 1985).

How are T cells generated? The functional definition of a T cell is a lymphoid cell which recognises Ag in the context of either Class I or II MHC antigen presented on the surface of a cell. This definition, while being strict in its limitation of functional mature cells, provides no insight into the mechanisms which have generated this/these cell phenotype(s).

As previously stated (see Introduction), functional T receptors are constructed of heterodimers produced from individual subunits, whose genomic arrangement and ability to rearrange from the germ line configuration is similar to that of the immunoglobulin genes. The function of these genes and the rearrangement they undergo would seem to be to produce the largest possible receptor set, by generating variability via differential recombination and somatic mutation assisted by the enzyme Tdt and the increased combined receptor repertoire generated by different α, β and γ subunit elements.

Where do these gene rearrangements occur and what control regulates their generation?

From preliminary researches it appears that the sequence of rearrangement perhaps has two phases. From examination of CTL-p frequency, both for recognition of allogeneic and hapten modified self and the construction of chimeric mice, it would appear that Class I self recognition occurs pre-thymically (Morrissey et al, 1982) in CTL-p. Receptors for antigen also expressed pre-thymically (Chervenak et al, 1985) and b haplotype specific CTL-p development and responsiveness are not suppressed by thymic b haplotype Class I antigens, but Class I b haplotype antigens on haemopoietic cells do suppress responsiveness (Von Boehmer and Schubiger, 1984). This theory is supported by the work of Kast et al (1985) who also postulate that the thymus defines the Class II specificity of T cells not the specificity of Class I restricted T cells.

It is interesting to speculate that the γ gene rearrangement thus far seen only in CTL may contribute or be wholly responsible for a receptor for Ag and/or self MHC in the bone marrow during early ontogeny of T cells. However, if CTL-p are not suppressed at the level of intra-thymic development, then it is necessary to postulate that anti-self reactive CTL-p are eliminated in the bone marrow or periphery prior to entry into the thymus. One means of removal of anti-self CTL-p would be to trap cells bearing receptors which had high affinity for self components. Such a system would leave the remaining receptor population with low and zero self affinity receptors and as such would account for non-self restricted populations (perhaps the basis of NK activity) and weak self + X (perhaps the basis of self Class I + Ag restricted and allospecific reactivity).

It is also possible that weak anti-self receptor reactivity is eliminated by a combination of tissue trapping, as above and elimination of such subsets by the thymus, due to their activation or partial activation in the periphery. However, if T cell receptors are generated at random, in an analogous fashion to B cells, to detect an unseen antigenic universe, then the greater the diversity of the receptor specificity the more likelihood there exists to generate a receptor for all possible antigens. It is impossible to say what proportion of receptors will show anti-self Class I specificity in comparison to cells bearing receptors which show no self reactivity although in adult animals anti-allotypic and hapten-modified self Class I CTL-p reactivity may account for up to 25% of all CTL-p reactivity detected (Reimann et al, 1985). This finding suggests that 75% or more of the receptors initially generated have very weak anti-self Class I reactivity if any.

It is thought that T lineage committed precursor cells are generated in the bone marrow, perhaps under regulatory signal molecules generated in the thymus which influence the precursors to migrate to the thymus. If the vast majority of migrants show low or zero self recognition, what do they recognise? It is perhaps prejudicial to describe early structures on pre-T cells as receptors and it may be informative to think of them as attachment points specific for other molecules whereas a true receptor is a molecule which binds another molecule with a resultant phenotypic change in the cell bearing the receptor molecule. The generation of a molecular binding site on bone marrow emigrating pre-T cells may explain the generation of anti-Class I recognising precursors, but is does not address the problem of the generation of the regulatory subsets of helper and suppressor T cells.

Once a pre-T cell enters the thymus it must, because of its intracellular purine catabolic enzymes, find itself in a particularly hostile environment, where the selective processes are greater. If elimination of self reactive pre-T cells is as postulated in the bone marrow by strength of binding by the lymphocyte binding site to a self-determinant, then the eventual efflux from the thymus would consist of low self-reactive and zero self-reactive abilities. If, on the other hand, tight binding of self determinants conferred survival advantage, the net result would be to expand reactivity to self molecules.

However, recent work has shown that the T cell receptor genes are rearranged and expressed non-randomly with γ chain mRNA being detected prior to β chain rearrangement, which precedes α chain expression during foetal ontogeny (R. Haars, unpublished data; Raulet et al, 1985). Using foetal thymocytes/BW5147 cell hybridomas these

findings have been confirmed showing some hybrids exhibit only γ rearrangement and others with γ and β genes rearranged.

Recent data on intra-thymic T cell precursors has defined a minor thymic subset (<2-4% total) which exhibits the Lyt-2$^-$ L3T4$^-$ phenotype and exhibits a dull staining for Lyt-1 and 20-30% give rise to Lyt2$^+$, L3T4$^+$ after 20-24 hours in vitro (Fowlkes et al, 1985). These dull Lyt-1 cells express β chain mRNA but not α chain mRNA and in hybrids of these cells a proportion do not exhibit β chain rearrangement (Samelson et al, 1985). This subset shows heterogeneity in that it is capable of repopulating both cortical and medullary areas. Only 20-30% of the cells differentiate in vitro, and there are un-rearranged and rearranged β chain genes, with heterogeneous expression of at least eight cell surface antigens. Another surface antigen phenotype em erged from this analysis: a bright Lyt-1$^+$, Lyt-2$^-$, L3T4$^-$ population which constituted <1% of the total but no data concerning the state of TcR genes was supplied.

Another group attempting to define the thymocyte precursor used the 95,000 Mr cell surface glycoprotein Pgp-1 which is present on most or all prothymocytes of the bone marrow and foetal thymocytes (Lesley et al, 1985a) but only on a few percent of adult thymus cells (Trowbridge et al, 1982). Most of these precursor cells (0.5% total), isolated by treatment of thymocytes with anti-Thy-1 antibody and complement, were shown to have the β TcR genes in germ line un-rearranged state and expressed little or no Thy-1 antigen (Trowbridge et al, 1985).

It should be noted that the Pgp-$^+$ Thy-1 low or Thy-1$^-$ showed only transient thymus repopulation and are heterogeneous (Lesley et al, 1985b) and that the heterogeneous Lyt-1 dull Lyt-2$^-$ L3T4$^-$ cells showed limited capacity for self renewal in vivo (Fowlkes et

al, 1985). While these subsets constitute 3% and 0.5% of the total lymphoid content of the thymus respectively, they are claimed to be precursors of more mature thymocytes, with heterogeneity of TcR gene rearrangement. If however 1% or less of all emmigrant prothymocytes survive transit through the thymus then most of these precursors must be destined to die in situ.

However, when the surface antigen phenotypes of thymocytes is considered several phenomena are observed. Foetal thymocytes (13 day gestation) express large amounts of IL-2 receptors while the proportion of cells expressing Thy-1, Lyt-1 and Lyt-2 is low. As gestation time increases the proportion of IL-2 receptor positive ($IL\text{-}2R^+$) cells decreases and Thy-1, Lyt-1 and Lyt-2 expression increases with the $IL\text{-}2R^+$ cells by day 16-19 are found predominatly in the outer cortex (Takacs et al, 1984). However, more recent data has demonstrated that by day 14-15 the majority of foetal thymocytes are $IL\text{-}2R^+$ $Thy1^+$, but Lyt^- and $L3T4^-$ and a subset of $IL\text{-}2R^+$ cells would proliferate in response to recombinant IL-2 (Hardt et al, 1985). These findings have been confirmed by other workers showing that at day 15, 66% of thymocytes were $IL\text{-}2R^+$ which decreased to 2% in adult mice (Ceredig et al, 1985; Raulet, 1985). When cells bearing the Lyt-2 and L3T4 antigens are eliminated, 51% of the $Lyt\text{-}2^-$ $L3T4^-$ phenotype are positive for IL-2R. The amount of IL-2R expression in foetal thymocytes is lower than in peripheral T cells and both high and low affinity receptors are seen, as in the periphery, but they are of lower K_D than the peripheral T cell. Even when $Lyt\text{-}2^-$ $L3T4^-$ thymocytes are exposed to high concentrations of IL-2 there is only marginal proliferation (Von Boehmer et al, 1985). When histological location of $Lyt2^-$ L3T4 $IL\text{-}2R^+$ cells is performed these cells appear scattered randomly throughout the cortex and medulla.

They are larger than most thymocytes and express high levels of Thy1 (Ceredig et al, 1985; Raulet, 1985). IL-2 and CON-A together provoked a vigorous proliferative response and splenic accessory cells could not substitute for either CON-A or IL-2 or both (Raulet, 1985; Teh and Ho, 1985). This co-stimulatory effect of IL-2 and CON-A is also seen in thymocyte responses to IL-1. Except at very high concentrations of IL-1, there is proliferation of unfractionated thymocytes but proliferation can be achieved at much lower levels if CON-A or PHA is also added (Wood et al, 1985).

When Lyt-2$^-$ L3T4$^-$ cells are examined for expression of mRNA encoding the α, β and γ T cell receptor subunit genes, high levels of B and γ mRNA are detected. As gestational age increases the β gene mRNA remains constant but the γ gene mRNA decreases as the α gene mRNA increases. Raulet et al (1985) have suggested that a γ/B heterodimer may allow selection or suppression of cells intrathymically, but the γ gene product has yet to be isolated (Raulet et al, 1985).

The heterogeneity of 'precursor' pro-thymocytes raises doubts as to whether there is a single cell surface antigen phenotype, which defines a single subset of bone marrow-derived T committed stem cells, or a range of thymus homing pre-T cells in various states of differentiation and receptor expression. This contention is supported by the varying states of T receptor gene rearrangement in a single surface antigen phenotype and the apparent surface antigen phenotype heterogeneity of the cells which evolve from a single surface antigen phenotype (see above).

From the metabolite exchange experiments it is obvious that some of the surface antigens expressed on MEC$^+$ cells could be classed

as exclusive, i.e PNA^+, $Lyt\text{-}1^+$, $Lyt\text{-}2^-$ and Ia^-, but this exclusivity breaks down when the Thy-1 and H-2 Class I phenotypes are considered. It could be argued that the low Thy-1 and $Thy\text{-}1^-$ are in fact part of a continuum from $Thy\text{-}1^-$ to Thy-1 high expression and the H-2 Class I phenotype reflects a similar transition. From surface antigen phenotyping of foetal thymocytes, it is distinctly possible that the thymus migrating cell is MEC^+ $Thy\text{-}1^-$ and expresses Thy-1 intrathymically, interacts with the stroma and/or gains Class I MHC expression before, during, or after stromal association and becomes MEC^- after Lyt-1 expression, prior to Lyt-2 expression. However, it could be argued that the MEC^+ phenotype is present at differing stages of expression of this series of surface antigens and that some cells expressing the same surface antigen phenotype are MEC^+ while other cells sharing the same phenotype are MEC^-.

The other possibility is that lymphoid cells are permanently MEC^+ from stem cell to fully differentiated effector cells and that the peak incidence of detectable MEC^+ cells in weanlings coincides with maximum thymocyte cellularity in the thymus and thus reflects maximum range or plasticity of receptor specificity for stromal cells permitting gap junction formation. In foetal and neonatal thymocytes the range of receptors is lower, due to lower cell numbers but this argues against receptor plasticity, as plasticity in receptor binding would throw up more MEC^+ cells than observed. In the post-weanling and adult thymus cells, MEC^+ cells may be harder to detect, as the MEC^- phenotype may predominate, or the specificity of the receptors is more restricted to the phenotype of the host stromal cells and interactions between MEC^+ thymocytes and the 'foreign' stromal cells is greatly reduced.

As previously stated, reticulum cells have been shown to secrete IL-1 (Papiernik and Homo-Delarche, 1983), which can cause proliferation of thymocytes, but only at high concentrations. If most immature thymocytes bear IL-1 and IL-2 receptors, secretion of IL-1 will only affect cells which are close to thymic macrophages and reticulum cells. If, on the other hand, the immature thymocytes bear receptors which can bind to epitopes on the surface of the macrophage or reticulum cell, then the interaction will stimulate the thymocyte in the same manner as the action of PHA or CON-A and the effect of the secreted IL-1 will be much amplified, causing proliferation of the bound thymocyte.

However, if the interaction of the thymocyte is strong, then the bound thymocyte may be engulfed and devoured, thus eliminating cells whose receptor only 'sees' self, whereas weak binding cells will gain the benefits of activation and IL-1 secretion and clonally expand. Strong binding thymocytes which have been phagocytosed, will be broken down and their surface antigens, including anti-self receptors, may be placed on the surface of the macrophage or dendritic cell, which could in turn act as an antigen presenting cell, with an anti-self receptor being presented on the macrohpage surface in the context of Class I or Class II. Recent work has suggested that the I-J molecule asociated with suppressor cell activity may be the 'internal image' of a Class II molecule, ie a receptor which binds a receptor which has specificity for Class II determinants (Sumida et al, 1985; Uracz et al, 1985). Other thymocytes may bind to reticulum cells and macrophages, which have taken up exogenous antigen and are presenting the antigen in the context of self elements, thus expanding cells which 'see' antigen and Class I or Class II to produce effector cytotoxic cells or helper cells.

It may also be the case that weak anti-self receptors on immature cells allow the thymocytes to closely appose themselves to the membranes of macrophages, reticulum cells and epithelial cells that gap junction formation can occur. When this happens the cells may not be activated, but rather they can rest in G_0 without the toxic accumulation of purine nucleotides, which would collect in G_0 cells that cannot discharge the build-up of purines to stromal cells. This phenomenon may account for the lack of T cells in SCID, as cells which attempt to discharge excess purines to stromal cells, find themselves coupled to thymic stroma which have equally high levels of purines due to their own defective ADA and PNP enzymes.

It is not known whether immature thymocytes require to enter G_0 to successfully rearrange their γ chain genes, but as γ chain rearrangement (and to a certain extent β chain rearrangement) occurs in the thymus, as is seen in the sequential expression of α, β and γ chain mRNA in the ontogeny of the organ. The interaction of immature thymocytes with epithelial cells via Class II molecules, may cause gap junction mediated mitotic arrest as in the case of the oocyte/cumulus cell complex. This in turn may allow the immature cells to rearrange their T cell receptor genes and allow mutation from the germ line by differential joining, diversity element insertion, and TdT mediated somatic mutation, to produce cell receptors which bind more weakly to the molecules which entrapped them prior to differentiation. This enables the mature cell to migrate to the periphery, where the receptor can interact with Class II and exogenous antigen. The events which occur in TNC are not known, but it is interesting to speculate that if mitosis is occurring, it is possible that more than one cell division occurs and that all progeny of mitosis are mutated away from direct self reactivity to varying reactivities

of a self + X receptor, thus increasing the size and range of the receptor set.

Thymocytes which can form gap junctions with stromal cells may gain all the advantages listed in the Introduction and earlier in the Discussion, such as synchronicity, resistance to sub-threshold stimulus, mitotic arrest, metabolic support, deliverance from environmental metabolic toxicity and increased mitotic potential by utilizing the products of enzymes of the host stromal cell. However, the ability to form gap junctions may have a role in mature lymphocyte function. The production of a response to an antigen, may involve the reactive lymphocytes forming gap junctions with antigen presenting cells via specific antigen and self molecules and result in the formation of germinal centres in the secondary lymphoid organs, allowing the reactive lymphocytes to proliferate more rapidly, due to metabolic support, than they normally would if isolated.

The frequency of MEC^+ cells observed in the thymus may be a gross underestimate. It is possible that all lymphocytes can form gap junctions, but the fine specificity of the receptors restricts gap junction formation to those cells which bear epitopes which the lymphocytes can bind to. If this is so, then neoplastic lymphoid cells which exhibit reduced or absent specificity may well be able to avoid cytotoxic agents by forming gap junctions with a host of stromal tissues, thus reducing the cytotoxic potential of lethal agents by sharing lethal agents with stromal tissue, thus lessening the damage caused to genetic material during mitosis. If lymphocytes can gain metabolic support by gap junction formation with stromal cells, this may enhance their growth and proliferation allowing them to reach far larger number, far faster than they could if dependent on their own metabolic processes.

However, most of this speculation remains to be proven and subsequent work in the ontogeny and control of lymphocyte differentiation and development will prove or disprove these flights of fancy.

BIBLIOGRAPHY

'Arbeit macht frei.'

('Work liberates.')

BIBLIOGRAPHY

Abbott, J., Doyle, P.J., Ngiam, K. and Olson, C.L. (1981) Cell. Immunol. 57: 237-250.

Abdou, N.I. and Abdou, N.L. (1972) Science 174: 446-447.

Acuto, O., Hussey, R.E., Fitzgerald, K.A., Protentis, J.P., Meuer, S.C., Schlossman, S.F. and Reinhertz, E.L. (1983a) Cell 34: 717-726.

Acuto, O., Meuer, S.C., Hodgdon, J.C., Schlossman, S.F. and Reinhertz, E.L. (1983b) J. Exp. Med. 158: 1368-1373.

Acuto, O., Fabbi, M., Smart, J., Poole, C., Protentis, J., Royer, H.D., Schlossman, S.F. and Reinhertz, E.L. (1984) Proc. Natl. Acad. Sci. 81: 3851-3855.

Allison, J.P., McIntyre, B.W. and Bloch, D.J. (1982) J. Immunol. 129: 2293-2300.

Altus, M.S., Bernstein, S.E., Russell, E.S., Carsten, A.L. and Upton, A.C. (1971) Proc. Soc. Exp. Biol. Med. 138: 985-988.

Anderson, E. and Albertini, D.F. (1976) J. Cell. Biol. 71: 680-686.

Archer, O.K., Sutherland, D.E.R. and Good, R.A. (1963) Nature 200: 337-339.

Arzania, R., Larsen, W.J. and Loewenstein, W.R. (1974) Proc. Natl. Acad. Sci. (USA) 71: 880-888.

Augustin, A.A. and Sim, G.K. (1984) Cell 29: 5-12.

Bach, M.A., Fournier, C. and Bach, J-F. (1975) Ann. N. Y. Acad. Sci. 249: 316-319.

Balch, C.M., Dougherty, P.A., Vogler, L.B., Ades, E.W. and Ferrone, S. (1978) J. Immunol. 121: 2322-2327.

Ballet, J.J., Insel, R., Merler, E and Rosen, F.S. (1976) J. Exp. Med. 143:1271-1276.

Baltimore, M.S. (1974) Nature 248: 409-411.

Barton, R., Goldschneider, I. and Bollum, F.J. (1976) J. Immunol. 116: 462-468.

Barton, R., Martiniuck, F., Hirschhorn, R. and Goldschneider, I. (1979) J. Immunol. 122:216-220.

Barton, R., Martiniuk, F., Hirshchhorn, R. and Goldschneider, I. (1980) Cell. Immunol. 49: 208-214.

Basch, R.S. and Berman, J.W. (1982) Eur. J. Immunol. 12: 359-364.

Beller, D.I. and Unanue, E.R. (1980) J. Immunol. 124: 1433-1440.

Bennedetti, E.L. and Emmlot, P. (1968) J. Cell Biol. 38: 15-24.

Berrih, S., London, J., Bonavida, B. and Bach, J.F. (1981) Imm. Meth. 41: 235-246.

Bessis, M. (1958) Rev. Haematol. 13: 8-15.

Bevan, M.J. (1975) J. Exp. Med. 142: 1349-1364.

Boersma, W.J.A., Betel, I., Daculsi, R., and van der Westen, G. (1981) Cell Tissue Kinet. 14: 179-196.

Boersma, W.J.A., Kokenberg, E., van der Westen, G. and Haaijman, J.J. (1982) Eur. J. Immunol. 12: 615-619.

Bollum, F.J. (1978) In: Advances in Enzymology 47: (Ed. A. Meister) pp347-374 J. Wiley & Sons, N.Y.

Bols, N.C., Kane, A. and Ringertz, N.R. (1979) Som. Cell. Genet. 5: 1045-1059.

Born, W. and Wekerle, A. (1982) Eur. J. Immunol. 12: 51-59.

Boyse, E.A. and Old, L.J. (1969) Annu. Rev. Genet. 3: 269-290.

Boyum, A. (1964) Nature 204: 793-794.

Brahim, F. and Osmond, D.G. (1973) Anat. Rec. 175: 737-746.

Bryant, B.J. (1972) Eur. J. Immunol. 2: 38-45.

Burrows, P.D., Lejeune, M. and Kearney, J.F. (1979) Nature 280: 838-841.

Caccia, N., Kronenberg, M., Saxe, D., Haars, R., Bruns, G.A.P., Goverman, J., Mallison, M., Willard, H., Yoshikai, Y., Simon, M., Hood, L. and Mak, T.W. (1984) Cell 37: 1091-1099.

Campbell, F.R. (1972) Amer. J. Anat. 135: 521-525.

Campbell, R. (1980) Anat. Record. 196: 101-111.

Cantor, H. and Boyse, E.A. (1975a) J. Exp. Med. 141: 1376-1389.

Cantor, H. and Boyse, E.A. (1975b) J. Exp. Med. 141: 1390-1399.

Cantor, H., Simpson, E., Sato, V.L., Fathman, C.G. and Herzenberg, L.A. (1975) Cell. Immunol. 15: 180-196.

Cantor, H. and Weissman, I.L. (1976) Prog. Allergy 20: 1-64.

Cantor, H. and Boyse, E.A. (1977) Immunol. Rev. 33: 105.

Carlsson, S.R. and Stigbrand, T.I. (1982) Eur. J. Biochem. 123: 1-7.

Carlsson, S.R. and Stigbrand, T.I. (1983) J. Immunol. 130: 1837-1842.

Casper, D., Goodenough, D.A., Makowski, L. and Phillips, W.C. (1977) J. Cell. Biol. 74: 605-628.

Ceredig, R., Glazebrook, A.L. and McDonald, R.H. (1982) J. Exp. Med. 155: 358-379.

Ceredig, R., Lowenthal, J.W., Nabholz, M. and McDonald, H.R. (1985) Nature 314: 98-100.

Chechik, B.E., Schrader, W.P. and Minowada, J. (1981) J. Immunol. 126: 1003-1007.

Chervenak, R., Cohen, J.J. and Miller, S.D. (1983) J. Immunol. 131: 1688-1692.

Chervenak, R., Moorhead, J.W. and Cohen, J.J. (1985) J. Immunol. 134: 695-698.

Chien, Y-h., Gascoigne, N., Kavaler, J., Lee, N.E. and Davis, M.M. (1984a) Nature 309: 322-326.

Chien, Y-h., Becker, D.M., Lindsten, T., Okamura, M., Cohen, D.J. and Davis, M.M. (1984b) Nature 312: 31-35.

Chiscon, M.O. and Golub, E.S. (1972) J. Immunol 108: 1379-1386.

Chui, D.H. and Russell, E.S. (1974) Dev. Biol. 40: 256-269.

Clark, S.L. (1968) J. Exp. Med. 128: 927-957.

Cohen, A., Lee, J.W.W., Dosch, H.M. and Gelfand, E.W. (1980) J. Immunol. 125: 1578-1582.

Coleman, M.S., Hutton., J.J. and Bollum, F.J. (1974) Nature 248: 407-409.

Conklyn, M.J. and Silber, R. (1982) Leuk. Res. 203: 10-18.

Coon, H.G. (1968) J. Cell. Biol. 39: 29a (No. 67).

Cooper, M.D., Peterson, R.D.A, South, M.A. and Good, R.A. (1966) J. Exp. Med. 123: 75-102.

Cooper, M.D. and Lawson, A.R. (1974) Sci. Amer. 231: (5) 59-77.

Cordier, A.C. and Heremans, J.E. (1975) Scad. J. Immunol. 4: 193-207.

Corsaro, C.M. and Migeon, B.R. (1978) Nature 272: 737-739.

Cox, R.P., Krauss, M.R., Balis, M.E. and Dancis, J. (1976) Exp. Cell. Res. 101: 411-414.

Cox, R.P., Krauss, M.R., Balis, M.E. and Dancis, J. (1979) Proc. Natl. Acad. Sci. 67: 1573-1579.

Cudkowitz, G., Upton, A.C., Shearer, G.M. and Hughes, W.L. (1964) Nature 201: 165-167.

Culvenor, J.G. and Evans, W.H. (1977) Biochem. J. 168: 475-481.

Dardenne, M., Pleau, J-M. and Bach, J-F. (1980) Eur. J. Imm. 10: 83-86.

Dardenne, M., Nabarra, B., Le Francier, P., Derrien, M., Choay, J. and Bach, J-F (1982) Proc. Natl. Acad. Sci. 79: 5370-5384.

David, J.C., Fedecka-Bruner, B., Mishal, Z., Vinson, D. and Rosenfeld, C. (1981) 11: 593-596.

Dennert, G., Swain, S.L., Waterfield , J.D., Warner, J.F. and Dutton, R.W. (1981) Eur. J. Immunol. 11: 62-68.

Despont J.P., Abel, C.A. and Grey, H.M. (1975) Cell. Immunol. 17: 487-493.

Dexter, T.M. and Lajtha, L.G. (1974) Br. J. Haematol. 28: 525-530.

Di Sabato, G., Moraes, E.K. and Altin, M. (1979) Cell. Imm. 44: 252-261.

Djaldetti, M., Bessler, H. and Rifkind, R.A. (1972) Blood 39: 826-841.

Dosch, H.M., Mansour, A., Cohen, A., Shore, A. and Gelfand, E.W. (1980) Nature 285: 494-496.

Draber, P. and Kieslow, P. (1981) Eur. J. Immunol. 11: 1-7.

Dulcibella, T., Albertini, D.F., Anderson, E. and Biggers, J.D. (1975) Develop. Biol. 45: 231-250.

Early, P., Huang, H., Davis, M., Calame, K. and Hood, L. (1980) Cell 19: 981-992.

Eckhardt, L.A. and Herzenberg, L.A. (1980) Immunogenetics 11: 275-292.

Epstein, M.L. and Gilula, N.B. (1977) J. Cell. Biol. 75: 769-787.

Erb, P. and Feldmann, M. (1975) J. Exp. Med. 142: 460-492.

Ezine, S. and Papiernik, M. (1981) Immunology 43: 535-540.

Farrar, W.L., Mizel, S.B. and Farrar, J.J. (1980) J. Immunol. 124: 1371-1377.

Fathman, C.G., Small, M., Herzenberg, L.A. and Weissman, I.L. (1975) Cell. Immunol. 15: 109-128.

Fernandez-Mejia, C., Debattisse, M. and Buttin, G. (1984) J. Cell. Physiol. 120: 321-328.

Fernbach, D.J. and Trentin, J.J. (1962) Proc. Int. Congress Haematol. 8th. 1: 150-153.

Finberg, R., Burakoff, S., Cantor, H and Benacerraf, B. (1978) Proc. Natl. Acad. Sci. (USA) 75: 5145.

Finberg, R., Greene, M.I., Benacerraf, B. and Burakoff, S.J. (1979) J. Immunol. 123: 1205-1209.

Finbow, M.E., Yancey, S.B., Johnson, R. and Revel, J-P. (1980) Proc. Natl. Acad. Sci. (USA) 77: 970-974.

Finbow, M.E. and Pitts, J.D. (1981) Exp. Cell. Res. 131: 1-13.

Finbow, M.E. (1982) Brit. Soc. Cell. Biol. Symp. 5: 1-37 In: Functional Integration of Cells in Animal Tissues (Eds. J.D. Pitts and M.E. Finbow) Cambridge University Press.

Finbow, M.E., Shuttleworth, J., Hamilton, A.E. and Pitts, J.D. (1983) EMBO J. 2: 1479-1486.

Fink, P.J. and Bevan, M.J. (1978) J. Exp. Med. 148: 766-775.

Fink, P.J., Weissman, I.L., Kaplan, J.S. and Kyewski, B.A. (1984) J. Immunol. 132: 2266-2272.

Fischer-Lindahl, K. and Hausmann, B. (1980) Eur. J. Immunol. 10: 289-298.

Flagg-Newton, J., Simpson, I. and Loewenstein, W.R. (1979) Science 205: 404-407.

Flint, O.P. and Ede, D.A. (1978) J. Cell. Sci. 31: 275-291.

Ford, C.E. and Micklem, H.S. (1963) Lancet i: 359-362.

Fowlkes, B.J., Edison, L., Mathieson, B.J. and Chused T.M. (1985) J. Exp. Med. 162: 802-822.

Fuduka, T. (1974) Virchows. Arch. Biol. 16: 249-270.

Fukumoto, T., McMaster, W.R. and Williams, A.F. (1982) Eur. J. Immunol. 12: 237-243.

Furshpan, E.J. and Potter, D.D. (1959) J. Physiol. 145: 289-325.

Furshpan, E.J. and Potter, D.D. (1968) Curr. Tops. Dev. Biol. 3: 95-127.

Gardner, R.L. and Rossant, J. (1976) Ciba Foundation Symposium 40: 5-25.

Garfield, R.E., Sims, S. and Daniel, E.E. (1977) Science 198: 958-960.

Garfield, R.E., Sims, S.M., Kannan, M.S. and Daniel, E.E. (1978) Am. J. Physiol. 235: 168-179.

Gascoigne, N., Chien, Y-N., Becker, D., Kavaler, J. and Davis, M.M. (1984) Nature 310: 387-391.

Gaunt, S.J. and Papaioannou, V.E. (1979) J. Embryol. Exp. Morph. 54: 263-275.

Germain, R. and Benacerraf, B. (1980) Springer Symp. Immunopathol. 3: 93-103.

Gey, G.O. (1952) Cancer Res. 12: 264.

Giblett, E.R., Anderson, J.E., Cohen, F., Pollara, B. and Meuwissen, H.J. (1972) Lancet ii: 1067-1069.

Giblett, E.R., Ammann, A.J., Wara, D.W., Sandman, R. and Diamond, L.K. (1975) Lancet i: 1010-1013.

Gillis, S., Union, N.A., Baker, P.E. and Smith, K.A. (1979) J. Exp. Med. 149: 1460-1476.

Gilmour, D.G., Theis, G.A. and Thorbecke, G.J. (1970) J. Exp. Med. 132: 134-147.

Gilula, N.B., Reeves, O.R. and Steinbach, A. (1972) Nature 236: 262-265.

Gilula, N.B., Fawcett, D.W. and Aoki, A. (1976) Dev. Biol. 50: 142-168.

Goldschneider, I., Ahmed, A., Bollum, F.J. and Goldstein, A.L. (1981) Proc. Natl. Acad. Sci. (USA) 78: 2469-2473.

Goodall, H. and Johnson, M.D. (1982) Nature 295: 524-526.

Goodenough, D.A. and Stockenuis, W. (1972) J. Cell. Biol. 54: 646-656.

Goodenough, D.A. and Gilula, N.B. (1974) J. Cell. Biol. 61: 575-590.

Goodenough, D.A. (1976) J. Cell. Biol. 68: 220-231.

Graham, C.F. (1977) In: Concepts in Mammalian Embryogenesis p315 (Ed. M. I. Sherman) M.I.T. Press, USA.

Grainger, R.M. and Wessels, N.K. (1974) Proc. Natl. Acad. Sci. (USA) 71: 4747-4751.

Greaves, M. and Janossy, G. (1973) Transpl. Rev. 11: 87-130.

Griepp, E.B. and Bernfield, M.R. (1978) Exp. Cell. Res. 113: 263-272.

Gregoire, K.E., Goldschneider, I., Barton, R.W. and Bollum, F.J. (1979) J. Immunol. 123: 1347-1352.

Haaijman, S.J., Micklem, H.S., Ledbetter, J.A., Dangl, J.L., Herzenberg, L.A. and Herzenberg, L.A. (1981) J. Exp. Med. 153: 605-614.

Hair, J. (1974) Proc. 1st. International Workshop on Nude Mice (Eds. J. Rygaard and C. O. Poulsen) Gustav Fischer Verlag, Stuttgart.

Hansen, T.H. and Levy, R.B. (1978) J. Immunol. 120: 1836-1840.

Hardt, C., Diamantstein, T. and Wagner, H. (1985) J. Immunol. 134: 3891-3894.

Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J. and Marrack, P. (1983) J. Exp. Med. 157: 1149-1169.

Hayes, C. and Hullett, D.A. (1982) Proc. Natl. Acad. Sci. (USA) 79: 3594-3598.

Hemmingian, E.J. and Linna, T.J. (1972) Int. Arch. Allergy 42: 693-710.

Henderson, D., Eibl, H. and Weber, K. (1979) J. Mol. Biol. 132: 193-218.

Hendrick, S.M., Cohen, D.I., Neilson, E.A. and Davis, M.M. (1984) Nature 308: 149-153.

Hertzberg, E.L. and Gilula, N.B. (1979) J. Biol. Chem. 254: 2138-2147.

Hiramatsu, K., Ochi, A., Miyatani, S., Segawa, A. and Tada, T. (1982) Nature 296: 666-670.

Hoessli, D., Bron, C. and Pink, J.R.L. (1980) Nature 283: 576-578.

Hood, L., Kronenberg, M. and Hunkapiller, T. (1985) Cell 40: 225-229.

Hopper, K. and Shortman, K. (1976) Cell. Immunol. 27: 256-273.

Hulser, D.F. and Peters, M.M. (1972) Exp. Cell. Res. 74: 319-326.

Hunig, T.R. and Bevan, M.J. (1980) J. Exp. Med. 152: 688-702.

Hunig, T.R. and Bevan, M.J. (1982) J. Exp. Med. 155: 111-125.

Hwang, W.S., Ho, T.Y., Luk, S.C. and Simon, G.T. (1974) Lab. Invest. 31: 473-487.

Ishii, K. and Green, H. (1973) J. Cell. Sci. 13: 429-439.

Irle, C., Piguet, P.F. and Vassalli, P. (1979) J. Exp. Med. 148: 32-45.

Jenkinson, E.J., van Ewijk, W, and Owen, J.J.T. (1981) J. Exp. Med. 153: 280-292.

Jenkinson, E.J., Franchi, L.L., Kingston, R. and Owen, J.J.T. (1982) Eur. J. Immunol. 12: 583-587.

Jerne, N.K. (1971) Eur. J. Immunol. 1: 1-9.

Jetten, A.M. and Jetten, M.E.R. (1979) Nature 278: 180-182.

Johnson, R.G. and Sheridan, J.D. (1971) Science 174: 717-79.

Jones-Villeneuve, E.V. and Phillips, R.A. (1980) Exp. Haematol. 8: 65-76.

Jordan, R.K., Robinson, J.H. and Waggott, E. (1979) J. Anat. 129: 871.

Jotereau, F.V., Houssaint, E. and Le Douarin, N.M. (1980) Eur. J. Immunol 10: 620-627.

Jotereau, F.V. and Le Douarin, N.M. (1982) J. Immunol. 129: 1869-1877.

Junker, S. (1982) Exp. Cell. Res. 139: 51-61.

Kadish, J.L. and Basch, R.S. (1976) J. Exp. Med. 143: 1082-1099.

Kamark, M.E. and Gottlieb, P.D. (1977) J. Immunol. 119: 407-415.

Kane, A.B. and Bols, N.C. (1980) J. Cell Physiol. 102: 385-393.

Kappler, J.W. and Marrack, P.C. (1976) Nature 262: 797-799.

Kappler, J.W., Skidmore, B., White, J. and Marrack, P. (1981) J. Exp. Med. 153: 1198-1214.

Kappler, J.W., Kubo, R., Haskins, K., Hannum, C., Marrack, P., Pigeon, M., Allison, J. and Trowbridge, I. (1983) Cell 35: 295-302.

Kapsenberg, M.L. and Leene, W. (1979) Exp. Cell. Res. 120: 211222.

Kast, M.W., de Waal, L.P. and Melief, C.J.M. (1985) J. Exp. Med. 160: 1752-1766.

Kefford, R.F. and Fox, R.M. (1982) Canc. Res. 42: 324-330.

Kelley, R.O. (1968) J. Exp. Zool. 172: 153-180.

Klein, G. (1975) N. Engl. J. Med. 298: 1353-1356.

Klinkert. W.E.F., Labadie, J.H., O'Brien, J.P., Beyer, C.F. and Bowers, W.E. (1980) Proc. Natl. Acad. Sci. (USA) 77: 54145418.

Konda, S., Stockert, E. and Smith, R.T. (1973) Cell. Immunol. 7: 275-289.

Kontiainen, S. and Feldman, M. (1977) Eur. J. Immunol. 7: 310-314.

Kruisbeek, A.M., Sharrow, S.O., Mathieson, B.J. and Singer, A. (1981) J. Immunol. 127: 2168-2176.

Kunz, P.C., Silverstone, A.E., McCaffrey, R.P. and Baltimore, D. (1975) J. Exp. Med. 141: 855-865.

Kyewski, B.A. and Kaplan, H.S. (1982) Proc. Natl. Acad. Sci. (USA) 79: 5646-5650.

Kyewski, B.A., Fathman, C.G. and Kaplan, H.S. (1984) Nature 308: 196-199.

Lafleur, L., Miller, R.G. and Phillips, R.A. (1973) J. Exp. Med. 137: 954-966.

Lampkin, J.M. and Potter, M. (1958) J. Nat. Can. Inst. 20: 1091-1111.

Lawrence, T.S., Beers, W.H. and Gilula, N.B. (1978) Nature 272: 501-506.

Leadbetter, M.L. and Lubin, M. (1979) J. Cell. Biol. 80: 166-182.

Ledbetter, J.A. and Herzenberg, L.A. (1979) Immunol. Rev. 47: 63-69.

Lepault, F. and Weissman, I.L. (1981) Nature 293: 151-154.

Lesley, J., Trotter, J. and Hyman, R. (1985a) Immunogenetics 22: 149-157.

Lesley, J., Hyman, R. and Schulte R. (1985b) Cell. Immunol. 91: 397-403.

Levitt, D. and Cooper, M.D. (1980) Cell 19: 617-621.

Levy, J.A., Weiss, R.M., Dirksen, E.R. and Rosen, M.R. (1976) Exp. Cell. Res. 103: 375-385.

Lieu, F.Y. (1981) Eur. J. Immunol. 11: 883-888.

Lindahl, P., Leary, P. and Gresser, I. (1974) Eur. J. Immunol. 4: 779-784.

Littlefield, J.W. (1966) Exp. Cell. Res. 41: 190-196.

Lo, C.W. and Gilula, N.B. (1979a) Cell 18: 399-410.

Lo, C.W. and Gilula, N.B. (1979b) Cell 18: 411-422.

Loewenstein, W.R. and Kanno, Y. (1964) J. Cell Biol. 22: 565-586.

Loewenstein, W.R. (1979) Biochem. Biophysic. Acta 560: 1-65.

Loor, F (1979) Immunology 34: 157-177.

Lord, B.I. (Ed) 1979 Brit. Soc. Cell. Biol. Symp. 2: 1-358.

Lord, B.I. (1981) Stem Cells 1: 140-147.

Lotan, R. (1980) Biochem. Biophysic. Acta 605: 33-91.

Low, T.L.K., Hu, S-K. and Goldstein, A.L. (1981) Proc. Natl. Acad. Sci. (USA) 78: 1162-1166.

Luriya, E.A. (1979) Bull. Exp. Biol. Med. 88: 1456-1458.

Lutz, C.T. (1981) J. Immunol. 127: 1156-1163.

Ma, D.D.F., Sylwestrowicz, T.A., Granger, A., Massaia, M., Franks, R., Janossy, G. and Hoffbrand, A.V. (1982) J. Immunol. 129: 1430-1435.

Ma, D.D.F., Sylwestrowicz, T., Janossy, G. and Hoffbrand, A.V. (1983) Immunol. Today 4: 65-68.

McDonald, C. (1982) Exp. Cell. Res. 138: 303-310.

McIntyre, B.W. and Allison, J.P. (1983) Cell 34: 739-746.

McIntyre, J.A., Gilula, N.B. and Karnovsky, M.J. (1974) J. Cell. Biol. 60: 192-203.

McIntyre, J.A., Pierce, C.W. and Karnovsky, M.J. (1976) J. Immunol. 116: 1582-1586.

McNutt, N.S. and Weinstein, R.S. (1970) J. Cell. Biol. 47: 666668.

McPhee, D. Pye, J. and Shortman, K. (1979) Thymus 1: 151-157.

Maden, M. (1982) Nature 295: 672-675.

Magnuson, T., Jacobson, J.B. and Stackpole, C.W. (1978) Dev. Biol. 67: 214-224.

Makowski, L. Caspar, L.D., Phillips, W.C. and Goodenough, D.A. (1977) J. Cell. Biol. 74: 629-645.

Malissen, M., Minard, K., Mjolsness, S., Kronenberg, M., Goverman, J., Hunkapiller, T., Prystowsky, M.B., Yoshikai, Y., Fitch, F., Mak, T.W. and Hood, L. (1984) Cell 37: 1101-1110.

Marin, G. and Littlefield, J.W. (1968) J. Virol. 2: 69-77.

Marmont, A. and Damasio, E. (1962) Proc. 8th. Congr. Eur. Soc. Haematol. 1: pp66-68.

Marrack, P., Shemankevitz, R., Hannum, K. and Kappler, J. (1983) J. Exp. Med. 158: 1635-1646.

Mathieson, B.J., Sharrow, S.O., Campbell, P.S. and Asofsky, R. (1979) Nature 277: 478-480.

Matter, A. (1979) Immunology 36: 179-190.

Matzinger, P. (1981) Nature 292: 497-501.

Matzinger, P. and Bevan, M.J. (1977) Cell. Immunol. 129: 1-12

Meera Khan, P. (1971) Arch. Biochem, and Biophysics 145: 470-483.

Metcalf, D (1960) Brit. J. Haematol. 6: 324-333.

Meuer, S.C., Acuto, O., Hussey, R.E., Hodgdon, J.C., Fitzgerald, K.A., Schlossman, S.F. and Reinhertz, E.L. (1983a) Science 222: 1239-1242.

Meuer, S.C., Acuto, O., Hussey, R.E., Hodgdon, J.C., Fitzgerald, K.C., Schlossman, S.F. and Reinhertz, E.L. (1983b) Nature 303: 808-810.

Migeon, B.R., Der Kaloustian, V.M., Nyhan, W.L., Young, W.J. and Childs, B. (1968) Science (NY) 160: 425-427.

Miller, J.F.A.P. (1965) Nature, 208: 1337-1338.

Miller, J.F.A.P. and Osoba, D. (1967) Physiol. Rev. 47: 437-520.

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A. and Gamble J. (1975) Proc. Natl. Acad. Sci. (USA) 72: 5095-5098.

Miller, R.A. and Stutman, O. (1982) Nature 296: 76-78.

Modak, M.J. (1979) Biochemistry 18: 2679-2684.

Monette, F.R. and Stockel, J.B. (1981) Stem Cells 1: 38-52.

Moor, R.M., Smith, M.W. and Dawson, R.M.C. (1980) Exp. Cell. Res. 126: 15-29.

Moore, M.A.S. and Owen, J.J.T. (1967) J. Exp. Med. 126: 715-726.

Morrissey, P.J., Kruisbeek, A.M., Sharrow, S.O. and Singer, A. (1982) Proc. Natl. Acad. Sci. (USA) 79: 2003-2007.

Nagy, Z.A., Elliott, B.E., Carlow, D.A. and Rubin, B. (1982) Eur. J. Immunol 12: 395-400.

Nieuwenhuis, P., van Honhuijs, C.E., Eggens, J.H. and Keuning, F.J. (1974) Immunology, 26: 497-507.

Nussenzweig, M.C. and Steinman, R.M. (1980) J. Exp. Med. 151: 1196-1212.

Nussenzweig, M.C., Steinman, R.M., Gutchinov, B. and Cohn, Z.A. (1980) J. Exp. Med. 152: 1070-1084.

Nussenzweig, M.C., Steinman, R.M., Unkless, J.C., Witmer, M.D., Gutchinov, B. and Cohn, Z.A. (1981) J. Exp. Med. 154: 168187.

Nyormoi, O., Sinclair, J.H. and Klein, G. (1973) Exp. Cell. Res. 82: 241-251.

Oliveira-Castro, G.M., Barcinski, M.A. and Cukierman, S. (1973) J. Immunol. 111: 1616-1619.

Orlic, D., Gordon, A.S. and Rhodin, J.A.G. (1965) J. Ultrastr. Res. 13: 516-542.

Owen, J.J.T. and Raff, M.C. (1970) J. Exp. Med. 132: 1216-1232.

Owen, J.J.T., Cooper, M.D. and Raff, M.C. (1974) Nature 249: 361-363.

Pahwa, R.N., Modak, M.J., McMorrow, J., Pahwa, S., Fernandes, G. and Good, R.A. (1981) Cell. Immunol. 58: 39-48.

Paige, C.J. (1978) J. Immunol. 121: 641-647.

Papiernik, M. and Homo-Delarche, G. (1983) Eur, J. Immunol. 9: 301-307.

Parkman, R., Gelfand, E.W., Rosen, F.S., Sanderson, A. and Hirschhorn, R. (1975) N. Eng. J. Med. 292: 714-719.

Paul, J. (1979) 'Cell and Tissue Culture' Churchill Livingstone, London.

Pazmino, N.H., McEwan, R.N. and Ihle, J.N. (1977) J. Immunol. 199: 494-499.

Peck, A.B., Alter, B.J. and Lindahl, K.F. (1976) Transplant. Rev. 29: 189-221.

Phillips, R.A. and Melchers, F. (1976) J. Immunol. 117: 1099-1103.

Phillips, R.A. and Miller, R.G. (1974) Nature 251: 444-446.

Phuc Li, H., Papiernik, M. and Dardenne, M. (1981) Clin. Exp. Imm. 44: 253-261.

Pitts, J.D. (1971) In: 'Growth Control in Cell Cultures' pp 89105 (Eds. G.E.W. Wolstenholme and J. Knight) Livingstone, London.

Pitts, J.D. (1972) In: 'Le Petit Colloquium on Cell Interactions' pp 277-285 (Ed. L.G. Silvestri) North-Holland Publications, Amsterdam.

Pitts, J.D. and Simms, J.W. (1977) Exp. Cell. Res. 104: 153-163.

Pitts, J.D. and Shaw, K. (1980) Cell. Biol. Int. Rep. 4: 758-760.

Pitts, J.D. and Shaw, K. (1981) Cell. Biol. Int. Rep. 5 Suppl. A: 45.

Pitts, J.D., Hamilton, A.E., Kam, E., Burk, R.R. and Murphy, J.P., Manuscript in Preparation.

Ploemacher, R.E.E., van'T Hull, E. and Vansoest, P.L. (1978) Exp. Haematol. 6: 311-320.

Pollack, G.H. (1976) J. Physiol. (London) 255: 275-298.

Porvaznik, M. and MacVittie, T.J. (1979) J. Cell. Biol. 82: 555-564.

Potter, D.D., Furshpan, E.J. and Lennox, E.S. (1966) Proc. Natl. Acad. Sci. (USA) 55: 328-336.

Preudhomme, J.L. (1978) Human Lymphocyte Differentiation: Its applications to Cancer, p 345. (Ed. Seron) North Holland, Amsterdam.

Pulvertaft, R.J.V. (1964) Lancet i: 238-240.

Raivio, K.O. and Hovi, T. (1979) Exp. Cell. Res. 116: 75-83.

Raulet, D.H. and Bevan, M.J. (1982) Nature 296: 754-756.

Raulet, D.H. (1985) Nature 314: 101-103.

Raulet, D.H., Garman, R.D., Saito, H. and Tonegawa, A. (1985) Nature 314: 103-107.

Reimann, J., Kabelitz, D., Heeg, K. and Wagner, H. (1985) J. Exp. Med. 162: 595-606.

Reisner, Y., Linker-Israeli, M. and Sharon, M. (1976) Cell. Immunol. 25: 129-134.

Revel, J-P. and Karnovsky, M.J. (1967) J. Cell. Biol. 33: 87-112.

Ringertz, N.R. and Savage, R.E. (1976) 'Cell Hybrids', Academic Press, New York.

Ritter, M.A. (1978) Immunology 24: 69-75.

Robertson, J.D. (1963) J. Cell. Biol. 19: 201-221.

Rock, K.L., Barnes, M.C., Germain, R.N. and Benacerraf, B. (1983) J. Immunol. 130: 457-462.

Roelants, G.E., London, J., Mayor-Withey, K.S. and Serrano, B. (1979) Eur. J. Immunol. 9: 139-145.

Ross, E.A.M., Anderson, N. and Micklem, H.S. (1982) J. Exp. Med. 155: 432-444.

Rothenberg, E. and Boyse, E.A. (1979) J. Exp. Med. 150: 777-791.

Rothenberg, E. (1982) J. Exp. Med. 155: 140-154.

Rothenberg, E. and Triglia, D. (1983) J. Immunol. 130: 1627-1633.

Royer, H.D., Acuto, O., Fabbi, M. Tizard, R., Ramachandram, K., Smart, J.E. and Rainhertz, E.L. (1984) Cell 39: 261-266.

Russell, E.S. (1979) In: 'Regulation of Haemapoiesis' 1: p 649. (Ed: A.S. Gordon) Appleton-Century-Crofts, New York.

Ryan, T.A., Joiner, B.L. and Ryan, B.F. (1981) Minitab Reference Manual and Minitab Students' Handbook, Duxbury, Mass.

Sacchi, N., Fiorini, G., Plevani, P., Badaracco, G., Breviario, D. and Ginelli, E. (1983) J. Immunol. 130: 1622-1626.

Saito, H., Kranz, D.M., Takagaki, Y., Hayday, A.C., Eisen, H.N. and Tonegawa, S. (1984a) Nature 309: 757-762.

Saito, H., Kranz, D.M., Takagaki, Y., Hayday, A.C., Eisen, H.N. and Tonegawa, S. (1984b) Nature 312: 36-39.

Samelson, L.E., Lindsten, T., Fowlkes, B.J., van den Elsen, P., Terhorst, C., Davis, M.M., Germain, R.N. and Schwartz, R.H. (1985) Nature 315: 765-768.

Sanderson, C.J., Hall, P.J. and Thomas, J.A. (1977) 196: 73-84.

Schaffner, Y., Muller, J., Hess, M.W., Cottier, H., Sordat, B. and Ropke, C. (1974) Cell. Immunol. 13: 304-312.

Schrader, J.W. and Schrader, S. (1978) J. Exp. Med. 148: 823-828.

Schrader, J.W., Goldschneider, I., Bollum, F.J. and Schrader, S. (1979) J. Immunol. 122: 2337-2339.

Schrader, J.W., Battye, F. and Scollay, R. (1982) Proc. Natl. Acad. Sci. (USA) 79: 4161-4165.

Scollay, R. and Weissman, I.L. (1980) J. Immunol. 124: 2841-2844.

Scollay, R. Jacobs, S., Jerabek, L., Butcher, E. and Weissman, I.L. (1980)a J. Immunol. 124: 2845-2853.

Scollay, R., Butcher, E.C. and Weissman, I.L. (1980)b Eur. J. Immunol. 10: 210-218.

Sellin, D., Wallach, D.F.H. and Fischer, H. (1971) Eur. J. Immunol. 1: 453-458.

Sellin, D., Wallach, D.F., Weltzien, H., Resch, K., Sprenger, E. and Fischer, H. (1974) Eur. J. Immunol. 4: 189-203.

Shaklai, M. and Tavassoli, M. (1978) Amer. J. Pathol. 90: 633-640.

Shaklai, M. and Tavassoli, M. (1979) J. Ultrastruct. Res. 69: 343-361.

Sharrow, S.O., Mathieson, B.J. and Singer, A. (1981) J. Immunol. 126: 1327-1335.

Sheridan, J.D., Finbow, M.E. and Pitts, J.D. (1979) Exp. Cell. Res. 123: 111-119.

Sheridan, J.D., Hammer-Wilson, M., Preus, D. and Johnson, R.G. (1978) J. Cell. Biol. 76: 532-544.

Sherwin, W.K. and Rowlands, D.T. (1974) J. Immunol. 113: 1353-1360.

Shields, J.W., Haston, W., and Wilkinson, P.C. (1984) Immunology 51: 259-268.

Shortman, K. and Jackson, H. (1974) Cell. Immunol. 12: 230-246.

Silverstone, A.E., Cantor, H., Goldstein, G. and Baltimore, D. (1976) J. Exp. Med. 144: 543-548.

Simpson, I., Rose, B. and Loewenstein, W.R. (1977) Science 195: 294-296.

Siu, G. Clark, S.P., Yoshikai, Y., Mallison, M., Yanagi, Y., Strauss, E., Mak, T.W. and Hood, L. (1984) Cell 37: 393-401.

Smets, L.A., van Rooy, H. and Homburg, C. (1980) Exp. Cell. Res. 123: 87-94.

Sodderhall, S. and Lindahl, T. (1975) J. Biol. Chem. 250: 8438-8444.

Sorrensen, G.D. (1961) Lab. Invest. 10: 178-193.

Spiegelman, M. (1976) In: 'Embryogenesis in Mammals' p 199 (Eds: K. Elliot and M. O'Connor) Elsevier.

Spurll, G.M. and Owen, F.L. (1981) Nature 293: 742-745.

S anford, K.K. (1948) J. Nat. Cancer Inst. 9: 229-246.

Steinman, R.M. and Witmer, M.D. (1978) Proc. Natl. Acad. Sci. (USA) 75: 5132-5136.

Steinman, R.M. and Nussenzweig, M.C. (1980) Immunol. Rev. 53: 127-147.

Stimson, W.H. and Crilly, P.J. (1981) Immunology 44: 401-407.

Stoker, M. and Macpherson, I.A. (1962) Virology 16: 147-151.

Stutman, O. (1978) Immunol. Rev. 42: 138-145.

Subak-Sharpe, H., Burk, R.R. and Pitts, J.D. (1966) Heredity 21: 342.

Subak-Sharpe, H., Burk, R.R. and Pitts, J.D. (1969) J. Cell. Sci. 4: 353-367.

Sumida, T., Sado, T., Kojima, M., Ono, K., Kamisaku, H. and Taniguchi, M. (1985) Nature 316: 739-741.

Sutherland, D.E.R., Archer, O.K. and Good, R.A. (1964) Proc. Soc. Exp. Biol. Med. 115: 673-676.

Swain, S.L. and Panfili, P.R. (1979) J. Immunol. 122: 383-391.

Swain, S.L., Dennert, G., Wormsley, S. and Dutton, R.W. (1981) Eur. J. Immunol. 11: 175-180.

Takacs, L., Osawa, H. and Diamantstein, T. (1984) Eur. J. Immunol. 14: 1152-1156.

Taniguchi, M., Tada, T. and Tokuhisa, T. (1976) J. Exp. Med. 144: 20-31.

Tavassoli, M. Ratzan, R.J., Maniatis, A. and Crosby, W.H. (1973) J. Reticuloendothelial Soc. 13: 518-526.

Tavassoli, M. (1977) Brit. J. Haematol. 35: 25-32.

Tavassoli, M. and Shaklai, M. (1979) Brit. J. Haematol. 43: 235-241.

Teh, H-S and Ho, M. (1985) J. Immunol. 134: 1653-1658.

Thuillier, L., Garreau, F. and Cartier, P. (1981) Cell. Immunol. 63: 81-90.

Tickle, C., Alberts, B., Wolpert, L. and Lee, J. (1982) Nature 296: 564-566.

Toolan, H.W. (1954) Cancer Res. 14: 660-666.

Trainin, N., Levo, Y. and Rotter, V. (1974) Eur. J. Immunol. 4: 634-637.

Trentin, J.J. (1971) Amer. J. Pathol. 65: 621-628.

Triglia, D. and Rothenberg, E. (1981) J. Immunol. 127: 64-68.

Trowbridge, I.S., Lesley, J., Schulte, R., Hyman, R. and Trotter, J. (1982) Immunogenetics 15: 299-312.

Trowbridge, I.S., Lesley, J., Trotter, J. and Hyman, R. (1985) Nature 315: 666-669.

Ulmer, A.J. and Flad, H-D (1979) J. Immunol. Meth. 30: 1-10.

Unanue, E.R., Perkins, W.D. and Karnovsky, M.J. (1972) J. Exp. Med. 136: 885-906.

Unwin, P.N.T. and Zampighi, G. (1980) Nature 283: 545-549.

Uracz, W., Asano, Y., Abe, R. and Tada, T. (1985) Nature 316: 741-743.

Van Ewijk, W., Rouse, R.V. and Weissman, I.L. (1980) J. Histochem. Cytochem. 28: 1089-1099.

Vogt, P, Erb, P. Keller, B. and Feldman, M. (1981) Eur. J. Immunol. 11: 377-381.

von Boehmer, H. and Schubiger, K. (1984) Eur. J. Immunol. 14: 1048-1052.

von Boehmer, H., Crisanti, A., Kisielow, P. and Haas, W. (1985) Nature 314: 539-540.

Wagner, H. Hardt, C., Heeg, K., Rollinghoff, M. and Pfizenmaier, K. (1980) Nature 284: 278-280.

Warner, A.E. and Lawrence, P.A. (1973) Nature 245: 47-49.

Wartiovarra, J., Lehtonen, E., Nordling, K. and Saxen, L. (1972) Nature 238: 407-408.

Weider, K.J., Araneo, B.A., Kapp, J.A. and Webb, D.R. (1982) Proc. Natl. Acad. Sci. (USA) 79: 3599-3603.

Weiss, C. (1980) 'Anatomy' p 323. McGraw-Hill, New York.

Weiss, L. (1970) Blood 36: 189-208.

Weissman, I.L. (1973) J. Exp. Med. 137: 504-510.

Wekerle, H., Ketelsen, U.P. and Ernst, M. (1980) J. Exp. Med. 151: 925-944.

Whitlock, C.A. and Witte, O.N. (1982) Proc. Natl. Acad. Sci. (USA) 79: 3608-3612.

Wilson, D.B. and Finta, L.A. (1979) Teratology 19: 337-344.

Witkop, C.J., White, J.G., King, R.A., Dahl, M.V., Young, W.G. and Sauk, J.J. (1978) Arch. Dermatol. 114: 1833.

Witkop, C.J., White, J.G., King, R.A., Dahl, M.V., Young, W.G. and Sauk, J.J. (1979) Am. J. Hum. Genet. 31: 414-427.

Wood, D.D., Bayne, E.K., Goldring, M.B., Gowen, M., Hamerman, D., Humes, J.L., Ihrie, E.J., Lipsky, P.E. and Staruch, M-J. (1985) J. Immunol. 134: 895-903.

Wortis, H.H., Nehlsen, S. and Owens, J.J. (1971) J. Exp. Med. 134: 681-692.

Wortman, R.L., Mitchell, B.S., Edwards, N.L. and Fox, I.H. (1979) Proc. Natl. Acad. Sci. (USA) 76: 2434-2437.

Yamamoto, N. and Bauer, G. (1981) J. Cell. Physiol. 109: 397-402.

Yamauchi, K., Murphy, D., Cantor, H. and Gershon, R.K. (1981) Eur. J. Immunol. 11: 905-918.

Yanagi, Y., Yoshika, Y., Leggett, K., Clark, S.P., Aleksander, I. and Mak, T.W. (1984) Nature 308: 145-149.

Yancey, S.B., Easter, D. and Revel, J-P. (1979) J. Ultrastruc. Res. 67: 229-242.

Yoffey, J.M. (1962) Lancet i: 206-211.

Zamboni, L. (1965) J. Ultrastruct. Res. 12: 509-524.

Zinkernagel, R.M., Dunlop, M.B.C., Blanden, R.V., Doherty, P.C. and Shreffler, D.C. (1976) J. Exp. Med. 144: 519-532.

Zinkernagel, R.M. (1976) J. Exp. Med. 144: 933-945.

Zinkernagel, R.M., Callahan, G.N., Klein, J. and Dennert, G. (1978a) Nature 271: 251-253.

Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Klein, P.A. and Klein, J. (1978b) J. Exp. Med. 147: 882-896.

Zinkernagel, R.M. (1978c) Immunol. Rev. 42: 224-239.

Zinkernagel, R.M. and Doherty, P.C. (1979) Adv. Immunol. 27: 52-177.

Zinkernagel, R.M., Althage, A., Waterfield, E., Kindred, B., Welsh, R.M., Callahan, G. and Pincetl, P. (1980) J. Exp. Med. 151: 376-399.