Studies on Cell Adhesion in Relation to Immune Responses.

A thesis submitted for the degree of Doctor of Philosophy by

Richard B. Gallagher.

Cell Biology Department.

August, 1984.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements.

My grateful thanks to Professor Adam Curtis for his interest, supervision and helpful discussion throughout this project. Thanks also to Dr. Caroline Birkby for guidance in the laboratory, to Dr. Jim Kerr for help with immunoblotting and Dr. Tony Lawrence for use of and help with fraction collector; to Mr. Andy Hart for photography and Mr. Mike McGrath for general assistance; to all the other members of the Cell Biology Department for their help and friendship; and to the Medical Research Council for funding me with a Research Studentship. Finally, thanks to my parents and to Ruth McKenzie for their great encouragement and help over the years.

Summary.

This work can be divided into three sections: the effects (i) of H-2D antigens on peritoneal exudate cell (PEC) adherence, (ii) of Ia antigens on PEC and lymphocytes and (iii) of reactive oxygen intermediates on lymphocyte activation.

H-2D antigen fragments can be attached to glass substates in an antigenically recognizable form and can affect the adhesion of PEC to the substrate. In all cases a significantly larger number (by t-test) of syngeneic than allogeneic cells adhere to fragments of the b, d and k allotypes. No differences between allogeneic and syngeneic adhesion could be found by interference reflection microscopy. When the average spread areas of cells were measured, they were found to be greater for allogeneic than for syngeneic combinations, although this could be due to contact inhibition of spreading in the latter. This work supports the suggestion that the MHC is involved in the modulation of cell adhesion.

The incubation of PEC in the supernatant of con A stimulated spleen cells resulted in adsorption of Ia antigens from the medium such that 20% of the cells were Ia positive at a 1/4 dilution of the supernatant. A purer Ia antigen preparation was made from LPS stimulated spleen cell supernatant using a monoclonal anti-Ia antibody column, and this was checked by immune blotting and SDS-PAGE. This preparation also adsorbs onto cells and is shown to influence mixed lymphocyte reactions. When B10 or Balb/b ($H\text{-}2^b$) cells were used as stimulators with B10.BR or Balb/k ($H\text{-}2^k$) as responders, the inclusion of Ia^k

antigens (ie. syngeneic to responders) significantly reduces the response. With B10.BR as stimulators and B10 as reponders, addition of Ia causes a significant increase in response although using Balb/k as stimulators and Balb/b as responders no difference is seen between presence and absence of Ia. The possibility of soluble Ia antigens modulating the immune response is discussed.

When peripheral blood mononuclear cells (PBM) are treated with potassium superoxide solution ($3x10^{-4}$ M) they are transformed to a similar degree as with mitogens. Stimulation of the monocyte component of PBM with zymosan-A, to induce O_2^-, also results in significant activation, a response which is blocked by superoxide dismutase. In an adhesion assay, treatment of PBM with 1mM potassium superoxide is shown also to induce a significant amount of adhesion. The results suggest that reactive oxygen intermediates are involved in lymphoctye activation, and the possible responsible species and mechanism of action is discussed.

Contents.

Abbreviations.

Ab	antibody
APC	antigen presenting cell
Asn	asparagine
BCGF	B cell growth factor
BGG	bovine gamma globulin
C3b	major cleavage product of third complement component
cDNA	copy DNA
CI	cytotoxicity index
con A	concanavalin A
CSF	colony stimulating factor
cys	cysteine
DCIP	dichlorophenolindophenol
d(Kd)	daltons (kilodaltons)
F_1	first filial generation
Fab	antigen binding fragment (of antibody)
Fc	crystallisable fragment (of antibody)
FCS	foetal calf serum
FITC	fluorescein iso-thiocyanate
H-2	murine MHC
$HO_2^{\cdot}$	hydroperoxyl radical (perhydroxyl ion)
H_2O_2	hydrogen peroxide
HEPES	4-[2-hydroxyethyl]-1-piperazine-ethanesulphonic acid
HLA	human MHC
Ia	I region associated antigen
IFN	interferon gamma
Ii	Ia associated invariant chain
IL 1	interleukin 1

IL 2	interleukin 2
IMF	interaction modulation factor
Ir	immune response gene
Ir-1	immune response-1 gene
LK	lymphokine
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
$^1O_2$	singlet oxygen
O_2^-	superoxide anion
OD	optical density
OH	hydroxyl radical
PBM	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PEC	peritoneal exudate cells
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate
PWM	pokeweed mitogen
ROI	reactive oxygen intermediates
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
[^{3}H]TdR	tritiated thymidine
TNP	trinitrophenyl
TRF	T cell replacement factor

CHAPTER 1.

INTRODUCTION.

In this thesis the importance of cell adhesion in the immune response is considered. The occurence of cell-cell adhesion in the immune system has been widely reported, for example in lymphocyte recirculation the cells must stick to the endothelium of the capillary venule (Bell, 1978a) in the face of the strong shearing force of the blood flow, and crawl into the lymphoid tissues. Also, during in vitro immune responses, lymphocyte-macrophage adhesion has been observed (Rosenthal, 1980) as has adhesion between cytotoxic T cells and their targets (Shortman and Goldstein, 1979).

Two types of theoretical model have been applied to analyzing cell-cell adhesion. In the first or non-specific model, adhesion is viewed as resulting from the general physical interactions between cells, for example van Oss and coworkers (1975) have suggested that cell-cell interactions may be similar to those between hydrophobic colloidal particles. In the second or specific model, adhesion is considered to arise from interactions between specific receptors and/or ligand molecules on the cell surface (Bell, 1978a). Of course even highly specific interactions such as those between antibody and antigen are made up of electrostatic, van der Waals and hydrophobic interactions so there is a certain arbritrariness in these distinctions.

The first two thirds of this thesis is concerned with the possible role of the major histocompatibility complex (MHC) in cell adhesion and the ability of class II (Ia) antigens to adsorb onto the surface of cells and alter their behaviour. Any MHC effects in modulation of adhesion are most likely to be of the

specific receptor-ligand type, in contrast to the effect of reactive oxygen intermediates (ROI), the subject of the final third of the thesis. The involvement of these highly reactive oxygen species in lymphocyte activation is described and shown to be accompanied by an increase in adhesiveness of the cells. An attempt is made to combine these two different types of mechanism into one model for lymphocyte activation. To this end, the structure and products of the major histocompatibility complex are of prime importance and will be considered first.

The Mouse Major Histocompatibility Complex.

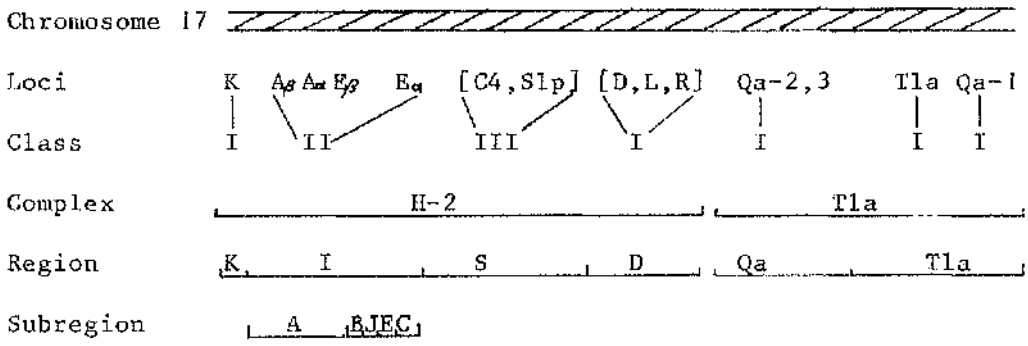
All mammals so far studied possess an MHC equivalent (Goetze, 1977). Those of mice and men, known as H-2 and HLA respectively, have been most extensively characterized and many structural features shown to be highly conserved between them.

At the genomic level all knowledge up until recently had been gained using indirect serological and cellular reactivity, but the recent advent of specific cDNA cloning is begining to yield spectacular results (see later). It has long been known that the H-2 complex is located in the middle of chromosome 17 (Klein, 1975) and measures approximately 1.5 recombination units in length which would, if the frequency of recombination was as for the rest of the genome, correspond to several million base pairs. Suppression of recombination between the T locus and the H-2 complex, however, indicate that this may not be the case (Bennet, 1980).

The currently accepted map (figure 1) divides the complex into

six main regions (K,I,D,Qa and Tla) which on the basis of phenotypic expression fall into three categories (Klein, 1979): class I antigens originally serologically defined are encoded in the K, D and L regions, class II(Ia) antigens, first charecterized by cellular reactivity, map to H-2I, and class III, components of the complement system are encoded in the H-2S region. Class III antigens will not be considered further here.

Figure 1. Genetic map of the mouse MHC.



from Steinmetz et al (1982b).

In the mouse six serologically defined class I antigens have been detected (Hansen et al, 1981) namely K, D, L, R, Qa and Tla (the last two of these are less well documented) and more possibly exist (Ivanyi and Demant, 1979). Although only one or two major products appear to be encoded from each region (Nairn et al, 1980) there are at least 36 distinct class I genes in 13 gene clusters in the mouse genome (Steinmetz et al, 1982a).

Each class I molecule consists of a large MHC-derived glycoprotein of 44,000 to 48,000 daltons associated noncovalently with β_2-microglobulin (Rask et al, 1974, Silver and Hood, 1974) a 12,000 dalton polypeptide which maps to mouse chromosome 2

(Robinson et al, 1981). The bulk of the molecule including the alloantigenic determinants (which reside on the polypeptide backbone (Nathenson and Cullen, 1974, Ploegh et al, 1981b) and require β_2-microglobulin for expression) is exposed on the extracellular side. The heavy chain spans the plasma membrane and concludes with a short C-terminal sequence which protrudes into the cytoplasm (Springer and Strominger, 1976, Walsh and Crumpton, 1977). This segment may play a role in transmembrane signalling via phosphorylation (Rothbard et al, 1980) and/or in interactions with cytoplasmic components such as actin (Koch and Smith, 1978).

Purification of class I molecules has been acheived with papain-cleaved heavy chains (Shimada and Nathenson, 1969) and detergent solubilized molecules (Schwartz and Nathenson, 1971) using standard protein fraction methods (Parham et al, 1977) in quantities large enough for protein sequencing (Nathenson et al, 1981). These studies have yielded information on the intramolecular organization of class I molecules (of several species) and allowed the study of the genetic interrelationship between these molecules. Striking similarities have been found in the extracellular region of the heavy chain, leading to its subdivision into three regions: N, the NH_2 terminal 90 residues includes an attachment site for a carbohydrate moiety at Asn_{86}; C1, the first disulphide loop region (residues 91-180) includes another carbohydrate attachment site at Asn_{176} and a disulphide bridge between Cys_{101} and Cys_{164} and C2, the second disulphide loop region (residues 181 to COOH terminus of papain cleavage) contains the second disulphide bridge between Cys_{203} and Cys_{259}.

Analysis of DNA sequences of cloned class I genes (Steinmetz et al, 1981) shows that these three regions are reflected precisely in the exon-intron structure of the genes. Unlike antibodies, no DNA rearrangement occurs in the expression of class I genes (Steinmetz et al, 1981) although there is convincing homology between antibody and class I genes, especially in the C2 domain. Structural homology between immunoglobulins, the second disulphide loop (Orr et al, 1979 using HLA-B7) and β_2-microglobulin has also been demonstrated at the protein level and it has been suggested that C2 and β_2-microglobulin associate together at the cell surface in a manner similar to immunoglobulin constant domains (Orr, 1982).

Studies of the 100 N-terminal residues of four H-2 molecules (two K and two D antigens) demonstrate a high degree of sequence homology, ranging between 74 and 87% with no apparent "K-ness" and "D-ness" (Nathenson et al, 1981). The differences between the molecules are non-randomly distributed into discrete regions, especially residues 61-83 and 95-99. These regions may contribute to the serologically detectable alloantigenicity of the molecules and are possibly functionally important.

Class I K, D, L and R antigens are integral components of the surface of almost all cells and are found in higher concentrations on lymphocytes than on other cells (Nathenson et al, 1981) whereas products of the Qa-1, -2, and -3 loci are resticted to expression on haemopoietic cells. Class II antigens, likewise, have a very limited tissue distribution (Ferrone etal, 1978). They are found most abundantly

on B lymphocytes, activated T lymphocytes, and antigen-presenting cells such as cells of the monocyte/macrophage line, Langerhans cells of the skin and dendritic cells of lymphoid and other organs (Nixon et al, 1982). Pober and Gimborne (1982) have also demonstrated class II antigen expression, inducible by phytohaemagglutinin, in vascular endothelial cells. Natural, as opposed to induced, expression of class II antigens by embryonic epithelial cells has also been reported (Jenkinson, et al, 1981). Ia antigens were shown to be present on normally developing mouse embryo thymic epithelium but not on that of 'nude' embryonic mouse thymus. This has lead to the suggestion that the epithelium is important in establishing the intrathymic enviroment for T cell differentiation.

Little is known unequivocally about the genotype of the class II antigens but again recombinant DNA technology is being applied. The two antigenically well characterized Ia antigens, I-A and I-E which are noncovalent heterodimers composed of α and β chains, are encoded in the I-A (A_α, A_β and E_β) and I-E (E_α) subregions. The current status of the other subregions ie. I-B, I-C and I-J, is unclear. Klein (1982) has argued that there is no biochemical evidence for the existence of either the I-E (Baxevanis et al, 1981) or I-C loci (Cullen et al, 1980) and studies at the genomic (Steinmetz et al, 1982b) level have supported this. Steinmetz et al (1981b) have further suggested that the I-A and I-E subregions may be contiguous and that the I-J product is possibly encoded outwith the I region altogether.

Both the α chain (molecular weight 31-34,000 daltons) and the β chain (molecular weight 26-29,000 daltons) are transmembrane glycoproteins (Cullen et al, 1976), 10 to 15% carbohydrate (Freed and Nathenson, 1978). There is strong evidence to suggest that the allodeterminants are contained within the protein (Cullen et al, 1976) but one group of investigators have raised the possibility that some Ia specificities lie within the carbohydrate portion (Sandrin et al, 1981). Both peptide mapping studies (Cook et al, 1979) and isoelectric focussing (Charron and McDevitt, 1980) have demonstrated that the β chain with and without carbohydrate is highly polymorphic and the α chain relatively invariant. From the few class II antigens which have been sequenced no homology has been found between the α and β chains (Orr, 1982) but homologies within α chains and β chains of different genotypes of the same species and between different species are very strong. It seems unlikely, therefore, that the smaller subunit (β) is derived from proteolytic cleavage of the larger (α) subunit. Steinmetz et al (1982b) have estimated that the I region contains only 4-6 loci and that 2 of these encode the α chains.

Intracellularly the α and β chains are asociated with a third polypeptide, Ii (Ia antigen-associated invarient chain) (Jones et al, 1978), a 31,000 dalton, nonpolymorphic, basic chain whose gene is not linked to the H-2 complex (Day and Jones, 1983). Ii is not associated with Ia antigens at the cell surface and its purpose intracellularly is possibly the transport and insertion

of Ia antigens (and perhaps other proteins) into the cell membranes. Koch et al (1982) have demonstrated Ii in the cell membrane independent of Ia antigens but its biological function is unknown.

In most of the work carried out on class II antigens B lymphocytes have been used. The few studies of Ia expression by T lymphocytes have yielded conflicting results, some groups finding the same pattern as on B cells (Koch et al, 1983) while others have found unique T lymphocyte determinants (Hiramatsu et al, 1982). This issue is important to the understanding of the role of the MHC in immune responses, the subject of the following section.

MHC Control of Immune Responsiveness.

In 1969 McDevitt and coworkers reported that variations in the capacity of inbred mice to make antibodies against branched chain polymers of amino acids mapped to a single gene or gene cluster, linked to the MHC, termed immune response-1 (Ir-1) genes. This was the first report of MHC involvement in the immune response, the full extent of which came to light in the early 1970's. Firstly, for T lymphocyte proliferation Rosenthal and Shevach (1973) demonstrated that T lymphocyte and antigen presenting cell (APC) had to have I-region compatibility. A similar requirement was shown for the interaction between T helper and B cells (Katz et al, 1973) and it was also found that specific cytolytic T lymphocytes could only lyse virally-infected (Zinkernagel and Doherty, 1974; Doherty and Zinkernagel, 1975) and TNP-modified

(Shevach et al, 1975) targets if target and responder cells shared the same H-2K and/or H-2D allele. These results suggest that T lymphocytes recognise antigen only in the context of a major histocompatibility antigen, a phenomenon which has since become known as MHC restriction.

A substantial body of evidence suggests that Ia antigens (ie class II antigens) are the products of the Ir genes. This includes the use of anti-Ia antisera and monoclonal antibodies to inhibit I-region-restricted T cell proliferation (Schwartz et al, 1978), T-dependent in vitro antibody responses (Frelinger, 1975) and mixed lymphocyte reactions (Greineder et al, 1976). It has been argued that the Ia antigens and the Ir gene products are merely closely linked structures on the cell surface so that binding of an Ia determinant by an antibody sterically blocks the adjacent Ir gene product. The finding that subregion mapping concords with the specificity of blocking antisera (Schwartz et al, 1978) argues against this.

Serological evidence of a similar nature has been put forward to demonstrate H-2K and D antigens as the restriction elements in cytotoxic T cell responses (Koszinowski and Ertl, 1975); antibodies against K and D determinants but not against Ia inhibited these responses. Using $(AxB)F_1$ animals Zinkernagel and Doherty (1975) showed that this reaction was not based on self recognition by the molecules. If this were the case individual T cells (which express K and D antigens of both parents) would be expected to lyse virally infected cells of both A and B haplotypes. In fact, separate populations of anti-A and anti-B

effectors were found, suggesting a complementary recognition system. Two models of this recogition system have been proposed, the dual receptor and altered self theories. The first of these, dual recognition, states that T cells exhibit two seperate receptors one of which shows specificity for MHC antigens, the other for non-MHC antigens. This theory has problems in explaining alloreactivity where the target carries only foreign MHC alleles : it suggests that alloreactivity is a special case where only one receptor need be bound. Even more difficult to explain, somatic cell hybrids of two T cells of different restriction specificities, X+A and Y+B cannot recognize targets expressing Y+A or X+B (Kappler et al, 1981), contrary to prediction. These problems are comfortably dealt with by the altered self hypothesis which suggests that T cells bear a single receptor which is only capable of recognizing antigens associated with H-2 in such a way as to create new antigenic determinants. A major assumption in this model is that molecules can form associations, perhaps transient, which exhibit new antigenic determinants (Matzinger, 1981). The transient nature of these weak associations would explain the lack of success in cocapping antigen and MHC antigens (Geib et al, 1977) although Koszinowski and Ertl (1975) have demonstrated that vaccinia virus infection causes alteration in H-2 antigens on the cell surface. The resolution of this issue may come when the precise nature of the T cell receptor is known. Studies using monoclonal antibodies raised against T cell hybridomas have identified "receptor material" on

both class I and class II MHC-specific cells (Acuto et al, 1983, Kappler et al, 1983). These two types of receptor have very similar gross structure, comprising two 40-43 kD polypeptides, α and β, each of which has a variable amino acid sequence. Recently two groups have reported the isolation of T cell-specific cDNA clones (Yanagi et al, 1984, Hedrick et al, 1984) which identify gene sets similar to, but distinct from, immunoglobulin genes. These code for a membrane-associated protein and in mice (Hedrick et al, 1984) are found to have undergone rearrangement before expression. A high degree of variability was found throughout their length, suggesting perhaps, a large combining site capable of recognizing both foreign and MHC antigen (Williams, 1984). In a recent study of a murine T helper cell receptor (Chien et al, 1984) a remarkable resemblance to immunoglobulin genes was demonstrated in amino acid sequence, pattern of rearrangement and size and position of gene segents.

It is known that the thymus is the important organ in the generation of the T cell repertoire although there is conflicting evidence over whether or not the T cells of an individual are entirely self restricted. The possibility that restriction is established only by selective priming on APC is supported by experiments in which type A T cells, depleted of alloreactivity to type B cells, may be primed to antigen on type B APC and can respond to antigen only in association with type B APC (Heber-Katz and Wilson, 1975, Thomas and Shevach, 1977). However, using radiation induced bone marrow chimaeras ($F_1 \rightarrow A$), the F_1 T cells

are capable of interacting only with the host (type A) APC (Zinkerngel et al, 1978). The grafting of type A thymus into a thymectomized, irradiated and F_1 bone marrow reconstituted F_1 mouse resulted in the same thymus-controlled restriction of interaction (Zinkernagel, 1978). The demonstration of maturing T cells totally allogeneic to the thymus, yet emerging to recognize foreign thymus MHC products as self, demonstrates the versitility of the T cell repertoire, the generation of which remains a mystery. It is currently thought that T cell precursors in the thymus generate a large array of receptors for self MHC in association with virtually any other antigen, the clones reacting with self being depleted. If this is correct alloreactive T cells would be raised when allogeneic MHC products cross-react with self MHC plus foreign antigen (Schwarz et al, 1978).

Despite the inherent dangers of teleology many authors have speculated on the function of the MHC and its renowned polymorphism. Klein (1979) proposed that the interaction of antigen with MHC products serves to increase the specificity of the immune response, restricting at two levels rather than one. Another possibility is that the MHC prevents many potential autoimmune reactions (Treissman, 1981) against antigens either not present in the thymus or not present at the appropriate stage in ontogeny and therefore not recognized as self. Alternatively, forbidden clones of T cells may be produced during a normal immune response, perhaps by mutation of the responding cells. Treissman reasons that the possibility of an autoimmune response is much reduced if these antigens or antigen receptors have to

associate with MHC antigens in the cell membrane. Langman and Cohen (1981) propose that the MHC controls the type of response elicited : intracellular infections are much more likely to result in the association of antigen with H-2K or D antigens, which are ubiquitous, thus activating H-2K and D restricted cytotoxic T cells. On the other hand, in extracellular infections antigen will only be expressed with MHC products on phagocytic cells which if they express Ia antigens, may lead to the generation of a humoral response.

Any hypothesis concerning the function of the MHC must take into account its remarkable polymorphism. This has been attributed to the selective advantage of heterozygosity in the individual and consequently to polymorphism in the species (Klein, 1979). An example of how this advantage would be manifest would be where the combination of products of a particular haplotype with a particular virus is not seen as nonself. In this case the consequences for a monomorphic species might be catastrophic whereas in a polymorhic species some individuals will be responders. A mathematical treatment of the question by Treissman (1981) has concluded that the individual selection of heterozygosity is, in the H-2 system, an adequate explanation of the polymorphism and that the immunological advantage to the species is a fortunate coincidence. Bodmer (1972) suggests that the polymorphism may have evolved as a consequence of the necessity for cell-cell recognition during development and morphogenesis, histocompatibility antigens acting either as differentiation antigens or their 'recognizers'. As work

continues on the MHC more possible explanations of function and theories of polymorphism will almost certainly come to light.

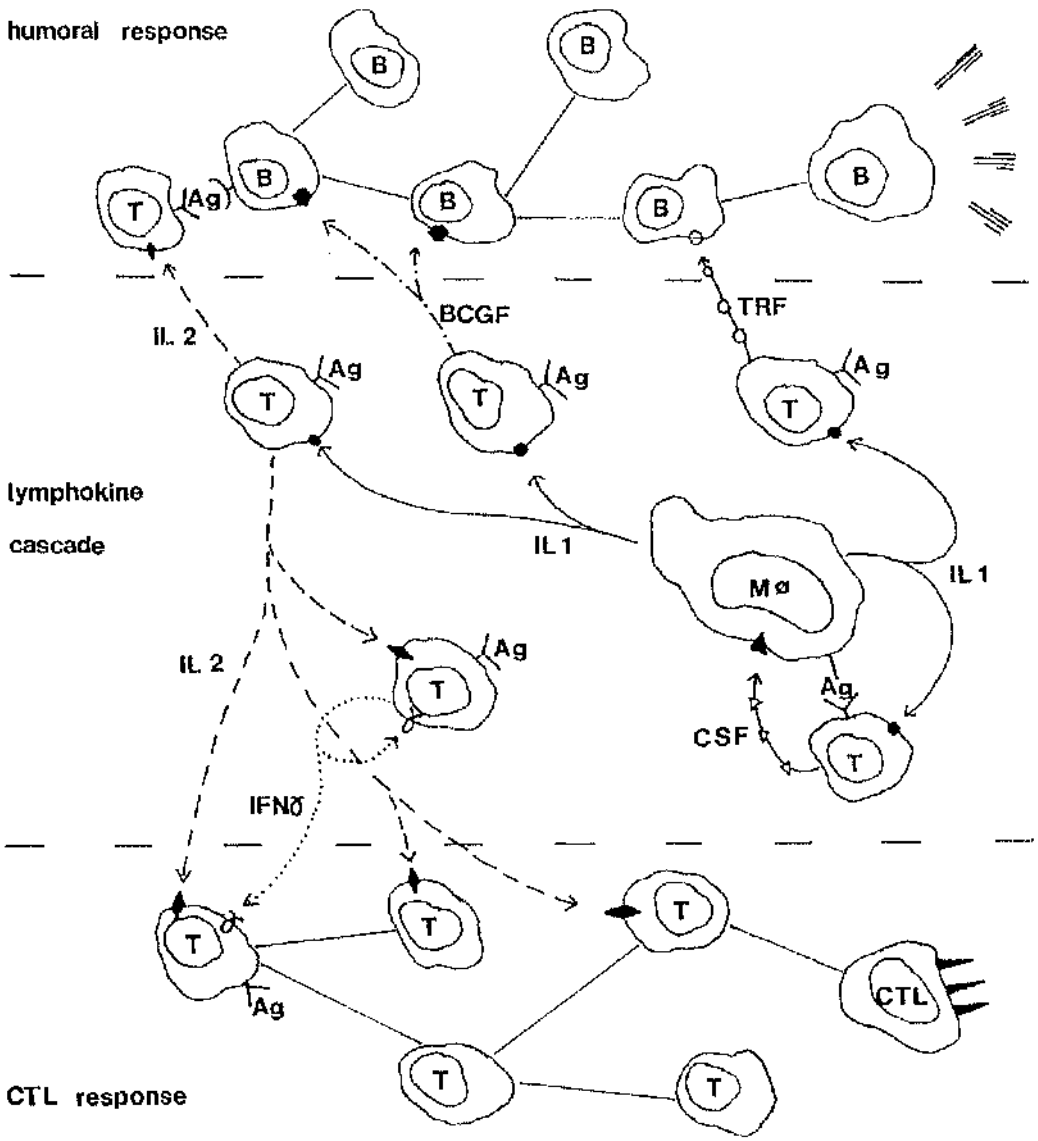
Cell Cooperation in the Immune Response.

The elicitation and control of immune responses involves complex interactions between different classes and subclasses of lymphocytes and between lymphocytes and antigen presenting cells (APC). The nature of the antigen itself profoundly influences the response. For example, particulate antigens provoke better responses than soluble ones and the valency of the antigen may influence T cell dependancy. There is variety also in the types of cells which will present antigen to T lymphocytes. Up until recently the macrophage alone was thought to have APC ability but it is now known that dendritic cells of lymphoid tissues are potent APC (Sunshine et al, 1983) as are B cell tumours and LPS-activated B cells (Chesnut et al, 1982). Dendritic cells, however cannot phagocytose, have few lysosomes and are poorly pinocytotic and consequently may be limited in the type of antigen they can handle. The mechanism of macrophage antigen handling has been studied using the organism Listeria monocytogenes (Ziegler and Unanue, 1981) as a large particulate antigen. Presentation to T cells, unlike antigen uptake, which occurs immediately, is only apparent after a 30-60 minute incubation period. Ziegler and Unanue suggest that this temperature-sensitive catabolism is an antigen processing event. They put forward the following scheme for antigen handling: firstly antigen interacts with the macrophage cell surface, perhaps via Fc or C3b receptors and is internalized within phagosomes.

Lysosome-phagosome fusion ensues, resulting in partial antigen degredation, and some fragments of the antigen are transfered to the cell surface by a proccess akin to exocytosis (Calderon and Unanue, 1974) where they associate with Ia molecules in such a manner as is recognized by T cells. Only Ia^+ macrophages can function as APC and Ia expression may be modulated by the products of activated spleen cells (Steeg et al, 1980), by the maturation state of the cell in question (Beller and Ho, 1982), and by the site it occupies (5-30% of peritoneal macrophages are Ia^+, 40-60% of splenic macrophages Ia^+) (Cowing et al, 1978).

The genetically restricted presentation of antigen is not the only APC signal upon which T cell activation is dependent, the low molecular weight antigen nonspecific peptide, interleukin 1 (IL 1) (Aarden et al, 1979) is also required. The major activity of IL 1 is thought to be the induction of synthesis and secretion of the T cell-derived mitogenic lymphokine interleukin 2 (IL 2) (Smith et al, 1980) which is important in promoting helper T cell proliferation (Watson, 1979). In fact a whole series of antigen specific and nonspecific amplification/suppression signals, mediated by T cell derived lymphokines are involved in lymphocyte activation. Dealing solely with the nonspecific factors, Farrar et al (1982) have proposed a lymphokine cascade (figure 2). This model predicts that while IL 1 and IL 2 are important in the generation of both humoral (top section) and cell-mediated (bottom section) immune responses they do not act directly on the effector cells but on the antigen-specific, genetically-restricted T helper cells. Less is known about the other

Figure 2. The Lymphokine Cascade in the Amplification of T and B Cell Responses. The central section depicts T-APC interaction giving rise to IL 1 and thence IL 2, BCGF (B cell growth factor) (Howard et al, 1982), TRF (T cell replacement factor) (Pickel et al, 1976) and IFN (interferon gamma) (Simon et al, 1977). CSF (colony stimulating factor) promotes macrophage Ia expression (from Farrar et al, 1982).



lymphokines in the cascade but they are all biochemically seperable factors.

There have been many reports of antigen-specific helper factors since the first description by Feldman and Basten in 1972 but, mainly because they have proven so difficult to purify, most of the work is phenomenology (reviewed by Tada and Okumara, 1979). Functionally these factors provide a reasonable proportion of the effect of helper cells themselves at very low concentrations (Howie and Feldman, 1977) which helps to explain why so little material is produced. Miyatani et al (1983) have biochemically analyzed an antigen-specific augmenting factor and found it to be a heterodimer of two distinct polypeptide chains each of 33,000 daltons, one carrying an immunoglobulin heavy chain determinant, the other an Ia-derived determinant. Both these determinants were unique to T cells. Biochemical charecterization of other helper factors will have implications not only in cell cooperaton but also in understanding the nature of the T cell receptor.

Less is known about specific supression of responses and again the factors involved are effective at extremely low concentrations. Like helper factors they are thought to be Ia^+, expressing especially products of the I-J subregion (Tada et al, 1976). The requirements for induction of a specific suppressive response appear to be quite different to those for a helper response: Feldman and Kontianen (1976) showed that very few, if any, macrophages were required and Dresser and Mitchison (1968) found that deaggregated proteins, for example bovine gamma globulin which are poorly taken up by macrophages, induce

suppression.

It is likely that in any immune response both helper and suppressor circuits are activated and it is the balance between these two which determines whether or not a positive response occurs and to what degree.

The MHC in Non-Immune Interactions.

The MHC has been associated with many non-immune physiological characteristics, including levels of complement components (Demant et al, 1973), androgen-sensitive organ weights (Ivanyi et al, 1972a), testosterone metabolism (Ivanyi et al, 1972b), susceptibility to viral leukaemogenesis (Lilley, 1971) and liver cAMP levels (Meruelo and Edidin, 1974). Since MHC antigens exert their effects at the cell surface, several groups of workers have studied the effects of H-2 type on cell adhesion using lawn collection assays. Using mouse embryo fibroblasts Bartlett and Edidin (1978) found that the H-2 complex modified adhesion, and the haplotype of the monolayer was all important. However, the suspended cells had not been allowed to recover from trypsinization. Zeleny et al (1978) concluded, from experiments using bone marrow or peritoneal cell monolayers and bone marrow or lymph node cell suspensions, that collection of cells in syngeneic combinatons was significantly greater than allogeneic although Pecknicova et al (1981), while finding that H-2 had an effect, could show no straight adhesive advantage of syngeneic over allogeneic combiations using the same assay system. From these experiments it was thought more likely that H-2 antigens are

closely linked to, or have a modifying effect on the receptors involved in cell adhesion rather than H-2 constituting the receptor itself.

Using a different assay system, lymphocyte aggregaton, Curtis (1978) and Curtis and de Souza (1975) have demonstrated specific soluble mediators of adhesion termed interaction modulation factors (IMF) which can be purified from cultures of both T and B lymphocytes. T cell derived IMF was subsequently shown to be a natural H-2D antigen fragment (Curtis and Davies, 1981) equivalent in nature to a papain fragment. In vivo, small quantities of this molecule had a marked effect on T and B cell circulation (Curtis et al, 1979) and in vitro altered not only lymphocyte adhesion but also fibroblast and epithelial cell adhesion (Curtis, 1982). Thus MHC antigen fragments affect adhesion of both allogeneic cells of the same tissue type and syngeneic cells of different tissues. This may be one line of evidence for Bodmers' suggestion (1972) that the main function of the MHC is control of cell-cell interactions.

A possible non-immunological function of the MHC is that of differentiation marking. Within the bone marrow for example, class II antigens appear on the very earliest progenitor cells but are absent from the pluripotent stem cell and from all the mature products other than the B lymphocyte and monocyte (Winchester and Kunkel, 1979). The differential expression of class II epitopes on tumour cell lines has led van Heyningen et al (1982) to suggest that class II molecules may act as malignant transformation markers and this is strongly backed up by the

finding that melanoma cells, derived from the neural crest, which is quite distinct embryologically from the lymphohaemopoietic system, also express Ia molecules.

Reactive Oxygen Intermediates.

In the reduction of molecular oxygen to water, radical intermediates including the superoxide anion (O_2^-), its conjugate acid the perhydroxyl or hydroperoxyl radical ($HO_2^\cdot$) and the hydroxyl radical ($^\cdot OH$) as well as other potentially harmful agents such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) may be formed.

O_2^- is produced in virtually all aerobic organisms (Halliwell *et al*, 1980) and, in appropriate circumstances can act as a powerful reducing agent by giving up its extra electron or as a weak oxidizing agent , becoming reduced to H_2O_2. The protonated form $HO_2^\cdot$ is a much more powerful oxidant so that dismutation is most rapid at acidic pH while *in vivo* O_2^- has a significant lifetime. Due to its low reactivity O_2^- is not thought to be directly responsible for many of the biological effects of reactive oxygen intermediates (ROI) (see later). These are probably mediated by O_2^--derived species of which $^\cdot OH$ and $^1O_2$ are the most likely candidates. It should be emphasized that $^1O_2$ has not been unequivocally shown to be derived from O_2^-, and is not a reduction product of molecular oxygen, but a high energy varient of it in which the two electrons unpaired in atmospheric (triplet) O_2 become paired. $^1O_2$ is a very reactive species, attacking especially double bonds in compounds which are inert to

atmospheric oxygen. Although $^1O_2$ has been implicated in the destruction of bacteria by phagocytes (Krinsky, 1974) no direct demonstration of $^1O_2$ production has been done for these cells. The generation of $^{\cdot}OH$ is thought to utilize O_2^- in an iron-catalyzed Haber-Weiss reaction (Halliwell et al 1974) :

Fe^{3+}-complex + O_2^- ⟶ Fe^{2+}-complex + O_2 (O_2^- as reductant)

Fe^{2+}-complex + H_2O_2 ⟶ Fe^{3+}-complex + $^{\cdot}OH$ + OH^- (Fenton-type reaction)

Net: $O_2^- + H_2O_2 \longrightarrow O_2 + {}^{\cdot}OH + OH^-$

The possible biological role of $^{\cdot}OH$ has been questioned by Bors et al (1980) who have pointed out that, due to its very high reactivity, there is a minimal chance of "free hydroxyl radicals" escaping from their site of generation and thus $^{\cdot}OH$ must be considered a "site specific radical" (Bachur et al, 1978). It is not yet clear, therefore, which of these species is more important biologically, and it is possible that they are involved in different phenomena.

The best studied enzyme systems for the generation of O_2^- are xanthine oxidase (Fridovitch, 1970) and the membrane-bound NAD(P)H oxidase of phagocytic cells (Baboir, 1978) which will be considered in more detail below. On exposure to stimulation by a wide variety of agents or by adherence (Berton and Gordon, 1983; Dahinden et al, 1983) phagocytes undergo a "respiratory burst" which begins with a sharp increase in oxygen uptake (Baldridge and Gerard, 1933). Using cytochalasin-B treated neutrophils Root and Metcalf (1977) showed that all the extra oxygen taken up during the respiratory burst was converted to O_2^- and that 80% of

this O_2^- was converted to H_2O_2 by dismutation. The identity of the enzyme responsible for the primary oxygen consuming reaction has been a controversial issue (Baboir, 1978). It is agreed that this enzyme catalyzes the reduction of molecular oxygen to O_2^- by a reduced pyrimidine nucleotide but whether the electron donor is NADH or NADPH has been the subject of dispute. Suzuki and Kakimura (1983) however, have recently shown by the addition of NADP and glucose-6-phosphate to neutrophils treated with Renex-30 (a detergent) that NADP oxidase is the primary oxidase of the respiratory burst, catalyzing the following reaction :

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

The oxidase is thought to be a multienzyme complex regulating the production of O_2^- through a series of linked redox reactions. Comparison of reduction peaks of activated and nonactivated cells has led to the identification of four components (Green et al, 1983a) namely DCIP (dichlorophenolindophenol) reductase, cytochrome c reductase, a chromophore 450-455 reductase and quinol dehydrogenase. The complex is dependent on Ca^{2+} and Mg^{2+} for full activity (Green et al, 1983b).

The dismutation of O_2^- is catalyzed by superoxide dismutases, reducing the chances of potentially deleterious ROI reactions. The first superoxide dismutase (SOD) charecterized, haemocuprein, was discovered in 1969 by McCord and Fridovitch, and, since then, two other types have been discovered (Keele et al, 1970, Yost and Fridovitch, 1973), the classification depending on the chelated metal (copper-zinc = haemocuprein, manganese and iron). They are all of approximately the same molecular weight (34-39,000

daltons) and are all composed of dimers of equal size. The reaction catalyzed by SODs is

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

The breakdown of H_2O_2, another potentially toxic product is catalyzed by the enzyme catalase :

$$H_2O_2 \longrightarrow H_2O + 1/2O_2 .$$

Although it is thought that the biological function of SOD and catalase is the rapid removal of harmful oxygen species, the roles of ROI in biology are many and various and not all deleterious. For example Esnouf et al (1980) have studied the production and utilization of O_2^- in the carboxylation of glutamyl residues of prethrombin and also a possible role for oxygen species has been suggested in prostoglandin biosynthesis (O'Brien and Rahimtula, 1976, van der Ouderra et al, 1977).

The peroxidation of lipids especially those with polyunsaturated fatty acids is one of the damaging features of ROI and O_2^- (Galeotti et al, 1981) and $^{\cdot}OH$ and $^1O_2$ (Ursini et al, 1980) have all been implicated. Peroxidation of lipids may bring about changes to both the structure and function of membranes : in erythrocytes lipid peroxidation contributes to ROI induced haemolysis (Chiu et al, 1976).

The most celebrated role of ROI is the antimicrobial activity of the phagocytic cells initiated, as described earlier, by the respiratory burst. It has been suggested (Baboir, 1978) that H_2O_2 (via oxidation of halide ions catalyzed by myeloperoxidase), $^{\cdot}OH$ and $^1O_2$ are all involved in the destruction of bacteria. The part played by the same species in inflammation, while less well

studied may be equally important. In fact the anti-inflammatory activity of SOD was appreciated long before its enzymic activity (Carson et al, 1973) and the involvement of ROI in the depolymerization of synovial fluid (McCord et al, 1974) and in the production of potent polymorph chemotactic factors (McCord et al, 1980) have been described.

The particular relevance of ROI to this thesis is their possible involvement in lymphocyte activation, as suggested by the work of several differant groups. Agro et al (1980) have demonstrated the biphasic reponse of lymphocytes to H_2O_2 : at very low concentrations (20nM) lymphocytes are stimulated (as shown by the uptake of tritiated thymidine) to the same extent as phytohaemagglutinin while at higher concentrations (500nM) H_2O_2 has an inhibitory, perhaps toxic, effect. Hydroxyl radicals may be involved in lymphocyte activation: Novogrodsky et al (1982) found that certain $^{\cdot}OH$ scavengers can inhibit lymphocyte proliferation in vitro. Thirdly, Wrogemann et al (1978 and 1980) have reported chemiluminescence in cultures of rat thymocytes immediately on exposure to concanavalin A. It is also of interest to note that the capacity of monocytes to secrete ROI is enhanced by lymphokines (Nakagawa et al, 1982) suggesting possibly some feedback control of ROI-mediated lymphocyte activation.

CHAPTER 2 .

MATERIALS AND METHODS.

1. Animals.

The mice used were initially bought from Olac animal suppliers, Bicester, England and subsequently bred and maintained in the animal house of the Southern General Hospital, Glasgow. Strains used were all of either Balb or B10 background ie. at non-H-2 loci. H-2 type of strains used is given with results. Within given experiments the animals used were age and sex matched.

2. Cell preparations.

(i). Human peripheral blood mononuclear cells (PBM) were obtained from normal, healthy laboratory personnel using Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of fresh heparinized blood. The cells were washed three times in Hanks-HEPES saline before use. Staining revealed these preparations to be 70-75% lymphocytes, 25-30% monocytes, viability > 90%.

(ii). Mouse splenocytes. Spleens were removed aseptically and squeezed through fine wire mesh (mesh size 400μm) to prepare a single cell suspension. The cells were washed twice in Hanks-HEPES saline before use.

(iii). Mouse peritoneal exudate cells (PEC). In all cases resident PEC, rather than activated, were used. Animals were killed, 3ml of cold Hanks-HEPES saline injected into the peritoneal cavity and the peritoneum massaged to suspend the cells. The abdominal skin was opened and a syringe with a wide diameter needle used to remove the medium. The cells, typical yield 1-2 x 10^6 per mouse, were washed twice in Hanks-HEPES

saline before use.

3. Media.

Hanks-HEPES saline (HHS).

NaCl	40g
KCl	2g
D(+)glucose	5g
0.5% phenol red	10ml
HEPES (4-[2-hydroxyethyl]-1-piperazine-ethanesulphonic acid)	11.92g
$CaCl_2.2H_2O$	0.93g
$MgCl_2.6H_2O$	1.00g

Made up to 5 litres with distilled water and pH adjusted to 7.5.

Phosphate buffered saline (PBS).

For x10 stock :

$NaH_2PO_4.H_2O$	20.5g
Na HPO .7H O	179.9g
NaCl	701.3g

Made up to 8 litres, pH 7.4.

RPMI 1640 medium.

x10 RPMI 1640 (Gibco)	10ml
HEPES water (20mM HEPES)	80ml
glutamine/penicillin/ streptomycin	2ml
bicarbonate ($NaHCO_3$, 7.5%)	0.5ml
2-mercaptoethanol, 0.1M	0.05ml

FCS (Gibco) 5ml/10ml

pH adjusted to 7.4 with 5M NaOH.

4. Preparation of H-2 class I antigen fragments.

H-2 K and D antigen fragments were kindly supplied by Professor A.S.G. Curtis. Briefly, his method of purification was by affinity chromatography on spleen cell culture supernatants using monoclonal antibody columns (Curtis and Davies, 1981). The fragments were eluted with diethylamine which was subsequently removed under vacuum. After sterilization, the biological activity of a small sample the material was checked and the remainder stored at 0°C until needed.

5. Attachment of class I antigen fragments to glass coverslips.

This was achieved using the method of Aplin and Hughes (1981). Firstly clean glass 13mm diameter coverslips were immersed in 3-aminopropyltriaethoxysilane, washed rigorously in water and incubated in 0.5% glutaraldehyde for 30 minutes at room temperature. The coverslips were then thoroughly washed in water before incubation for one hour at room temperature in H-2 fragment-containing solution (see above). After washing in PBS coverslips were ready for use.

6. Immunofluorescent stanining of coverslip-bound H-2 antigens.

To confirm that H-2 antigens had attached to coverslips (section 5 above), monoclonal antibodies 15.5.5 (anti-D^b) and 141.34 (anti-D^k) (American Tissue Type Collection, Maryland, USA) diluted 1000-fold were incubated with coverslips treated with H-

$2D^b$ and H-$2D^k$ antigen fragments. After two hours at room temperature, the coverslips were thouroughly washed in PBS and 1 in 40-diluted FITC-conjugated rabbit anti-mouse Fab (Nordic Immunologicals, Tilburg, The Netherlands) added and incubated for a further hour at room temperature. The coverslips were thoroughly washed and examined using a Vickers Photoplan fluorescence microscope fitted with an HBO 200W mercury arc lamp and appropriate barrier filters.

7. Assay of peritoneal exudate cell attachment to H-2-coated coverslips.

One million peritoneal exudate cells in a volume of 1 ml of RPMI 1640 plus 5% FCS were added to H-2—coated 13 mm diameter coverslips in 16 mm diameter Linbro multiwell tissue culture plates (Flow Laboratories, Irvine, Scotland) and incubated for 30 minutes at 37°C. Coverslips were then washed in PBS, fixed in formol saline and stained with Coomassie blue. The number of cells attached and total spread area of those cells was measured using a Quantimet image analyzing computer (Cambridge Instruments, Cambridge, England), ten fields being measured per preparation (1 field = 2.867×10^5 μm^2).

8. Ia antigen preparation.

The method used was similar to that of Nagy et al (1982). Mouse spleen cells were adjusted to 3×10^6 ml^{-1} in RPMI 1640 containing 10% FCS and 10$\mu g ml^{-1}$ lipopolysaccharide (LPS). Three day LPS blasts were resuspended at 6×10^6 cells ml^{-1} in RPMI 1640 containing 0.5% FCS, 0.01% Tween 80 and 1.4 units ml^{-1} aprotinin, a protease

inhibitor. After 8-10 days at 37°C the cells were removed by centrifugation. In some experiments, where indicated, this crude preparation was used, in others (Ia^k fragments) further purification steps were done: an immunoadsorbent column was made by conjugating an anti-Ia^k monoclonal antibody (anti-Ia^k specificity 17, Becton Dickinson Laboratory Systems, Mechelen, Belgium) to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Company, St. Louis, Missouri) (for method see Mishel and Dutton, 1980). The culture supernatant was applied to the column which was washed extensively with PBS. Ia antigens were eluted as a single peak using 0.05M diethylamine, pH 11.5 which was subsequently removed under vacuum. The preparation was checked by SDS-PAGE and immunoblotting (see section 9).

9. Electrophoresis and Immunoblotting of Ia antigens.

SDS-PAGE reagents and techniques used were as described by Laemmli (1970). Silver staining was done following the method of Oakley et al (1980). Immunoblotting of gels onto nitrocellulose paper was acheived using the method of Towbin et al (1979). The paper was treated firstly with 1 in 50 diluted anti-Ia^k antisera (Cedarlane Laboratories, Ontario, Canada) and secondly with ^{125}I-protein A (kind gift of Dr. Jim Kerr, Dept. of Cell Biology) for 6 hours. This was then vizualized by autoradiography, exposure of X-omat HX X-ray film (Kodak plc, London, England) for 3 days.

10. Iodination of Ia antigens and assay of attachment to PEC.

A small quantity of the Ia antigen preparation (500 µl) was

iodinated using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-gen, Pierce Chemical Co., Rockford, IL., USA) (Fraker and Speck, 1978) and a 50µl sample counted to determne activity.

10^6 PEC in 1 ml of media were allowed to adhere to 13mm diameter glasss coverslips in 16mm diameter Linbro multiwell tissue culture plates for thirty minutes at 37°C. They were then thoroughly washed with PBS to remove nonadherent cells and 0.5ml of PBS containing 50µl ^{125}I-conjugated Ia antigen added and incubated for a further 30 minutes at 37°C. The coverslips were again thoroughly rinsed and the amount of label bound determined using a Wilj 2001 gamma counter.

11. Complement lysis assay.

This assay utilized microtitre plates (Falcon Plastics, Becton - Dickinson, Grenoble, France). $2x10^4$ PEC were added to each well and allowed to adhere for 30 minutes at 37°C. The cells were then washed and media containing a dilution of Ia antigen preparation was added and the plates incubated overnight. After washing the plates thoroughly anti-Ia^k serum (Cedarlane Laboratories) diluted 1/200 in Hanks-HEPES was added and incubated for 60 minutes at 4°C. The cells were again washed thoroughly and resuspended in 1/100-diluted guinea-pig serum (Wellcome Reagents plc, Beckenham, England) as a source of complement. After 1 hours incubation at 37°C the complement was removed and the degree of lysis determined by trypan blue exclusion (0.2% trypan blue for 5 minutes, live cells exclude stain), the cytotoxicity index (CI) calculated as follows :

$$CI = \frac{\%\ lysis\ (Ab + C') - \%\ lysis\ (C'\ alone)}{100\% - \%lysis\ (C'\ alone)} \times 100\%$$

12. Mixed Lymphocyte Reaction (MLR).

Both stimulator and responder cells were mouse splenic mononuclear cells prepared on a Ficoll-paque density gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). Stimulators, resuspended at $4x10^5$/ml were treated with 25µg/ml of mitomycin C (Sigma) for 20 mins at 37°C and then washed three times in PBS-5%FCS. After adjusting both stimulators and responders to $2x10^5$ per ml, 0.5 ml from both preparations were mixed together in sterile, plastic, conical-bottomed test tubes (Sterilin, Teddington, England) and cultured for 96 hours. 1µCi (Amersham International plc, Amersham, England) of tritiated thymidine ([^{3}H]TdR) was added to each tube during the final four hours of culture before harvesting onto GF-A glass fibre filters (Whatman Chemical Seperation Ltd, Maidstone, England), washing with 10% trichloroacetic acid (TCA) (BDH Chemicals Ltd, Poole, England) and ethanol. The filters were placed in Pico-Fluor scintillation fluid (Packard, Zurich, Switzerland) and counted in a Packard Tricarb 300 scintillation counter. Triplicates of each treatment were assayed and, where appropriate, 20µl of Ia antigen preparation added at the begining of the culture.

13. Mitgenic Stimulation of Lymphocytes.

Five million human peripheral blood mononuclear cells (PBM) were cultured in sterile, conical-bottomed, plastic test tubes (Sterilin) for 96 hours with 1 µCi of [^{3}H]TdR added for the last

four hours before harvesting as for MLR (section 11 above). Mitogens were included throughout the culture period at the following concentrations: phytohaemagglutinin (PHA) (Sigma) 2μg/ml, concanavalin A (con A) (Sigma) 4μg/ml and pokeweed mitogen (PWM) (Sigma) 10μg/ml. When included in cultures, zymosan-A (Sigma) was added at 10 particles per cell (ie $5x10^7$ /ml), mannitol (BDH) at 50 mM, catalase (Sigma) at 0.1mg/ml and superoxide dismutase (SOD) (Sigma) at 0.1 mg/ml.

14. Treatment of lymphocytes with potassium superoxide.

$5x10^6$ human PBM were suspended in Hanks-HEPES containing freshly dissolved potassium superoxide (in solution superoxide has a half life of several seconds at millimolar concentrations so solutions were used immediately). After 1 hour at 37°C the cells were thoroughly washed and a proliferative assay performed as above (section 12). The reason for this long incubation period being that $^{\cdot}OH$ and possibly other superoxide-derived radicals have, potentially, a fairly long half life.

15. Measurement of cell adhesion.

10^6 PBM in 1ml of Hanks-HEPES were exposed to freshly made 1mM potassium superoxide and a cell adhesion assay was performed at 37°C in a Couette viscometer (Curtis, 1969) which provides a continuous, constant shear. Samples were removed at regular intervals and the number of particles (single cells or aggregates) counted using phase contrast microscopy to detect and a Quantimet 720 image analyzing computer to count the particles.

CHAPTER 3.

THE EFFECT OF H-2D ON PERITONEAL CELL ADHESION.

Introduction.

The possible involvement of the MHC in cell interactions was first suggested by Bodmer in 1972 and has been the subject of much research since (see introduction). The assays used, lawn collection and adhesion assays complicated the interpretation of the results by virtue of the fact that two interacting cell surfaces had to be considered. With the purification of H-2 antigen fragments (Curtis and Davies, 1981) and a method for attaching proteins to chemically modified glass surfaces (Aplin and Hughes, 1981) it has become possible to study the adhesion of cells to H-2 antigen coated substrates, considerably simplifying the system.

In this chapter the adhesion of mouse peritoneal exudate cells to H-2D antigen coated glass coverslips is reported.

Verification of the attachment of H-2D antigens to coverslips.

Before measurements of the effect of the H-2D on adhesion could be made, the binding of antigen to coverslips had to be verified and the optimal concentration of cells and length of incubation to be determined. Coverslips treated with $H\text{-}2D^b$ and $H\text{-}2D^k$ antigen preparations were subjected to indirect immunofluorescence analysis using monoclonal anti-D^b (15.5.5) and anti-D^k (141.34) antibodies followed by FITC-conjugated rabbit anti-mouse Fab antisera. The results shown in table 1 indicate that the appropriate antibodies pick out $H\text{-}2D^b$ and $H\text{-}2D^k$ treated coverslips, that there is no cross-reaction between these and no nonspecific fluorescence in controls. Positive fluorescence was weak but definite and taken as sufficient evidence for the

attachment of H-2D antigen fragments.

Table 1. Detection of H-2 antigen fragments on chemically modified surfaces.

Treatment of coverslip	Immunofluorescence using	
	15.5.5 (anti-D^b)	141.34(anti-D^k)
D^b (B10) prep.	+	–
D^k (Balb/k) prep.	–	+
FCS	–	–
PBS	–	–

+ = weak fluorescence

– = no fluorescence

Temporal and concentration calibrations of adhesion.

Ideally a checkerboard assay varying time of incubation over a range of concentrations would have been carried out but the yield of PEC (approximately 10^6 per mouse) made this impractical. To determine the optimal cell concentration PEC were incubated on FCS coated coverslips for 30 minutes at 37°C over the range 10^4 to $5x10^6$ per ml before fixing, staining and counting in the Quantimet image analyzing computer. Two parameters, the number of cells attaching and the average area of attached cells were measured, the results of which are shown in figure 3. This shows that at high concentrations of cell suspension a larger number of cells adhere with a much reduced spread area, possibly due to contact inhibition of spreading. To test this, the extent of cell-cell contact was assessed using the formula

Table 2.

Peritoneal exudate cells were incubated for thirty minutes at 37'C in multiwell tissue culture plates containing FCS-coated glass coverslips. The coverslips were washed in PBS, fixed in formol saline and stained with coomassie blue. The numbers of cells attached and the average spread area were measured using an image analyzing computer. The values shown are mean and standard deviations for 10 readings from each of duplicate preparations.

Table 2. Changes in number of cells adhering, area of those cells and number of cell-cell contacts with concentration of cell suspension.

conc. of suspension (cells/ml)	no. of cells adhering (per field)	av. area of cells (ppu)	no. of contacts per field	av.% cells making contacts
5×10^4	5 ± 1	712 ± 47	0.06	1.1
10^5	6 ± 3	808 ± 162	0.10	1.6
5×10^5	31 ± 4	745 ± 7	2.8	8.9
10^6	75 ± 13	747 ± 31	16	22
5×10^6	229 ± 52	465 ± 88	94	41

Results given as mean ± standard deviation

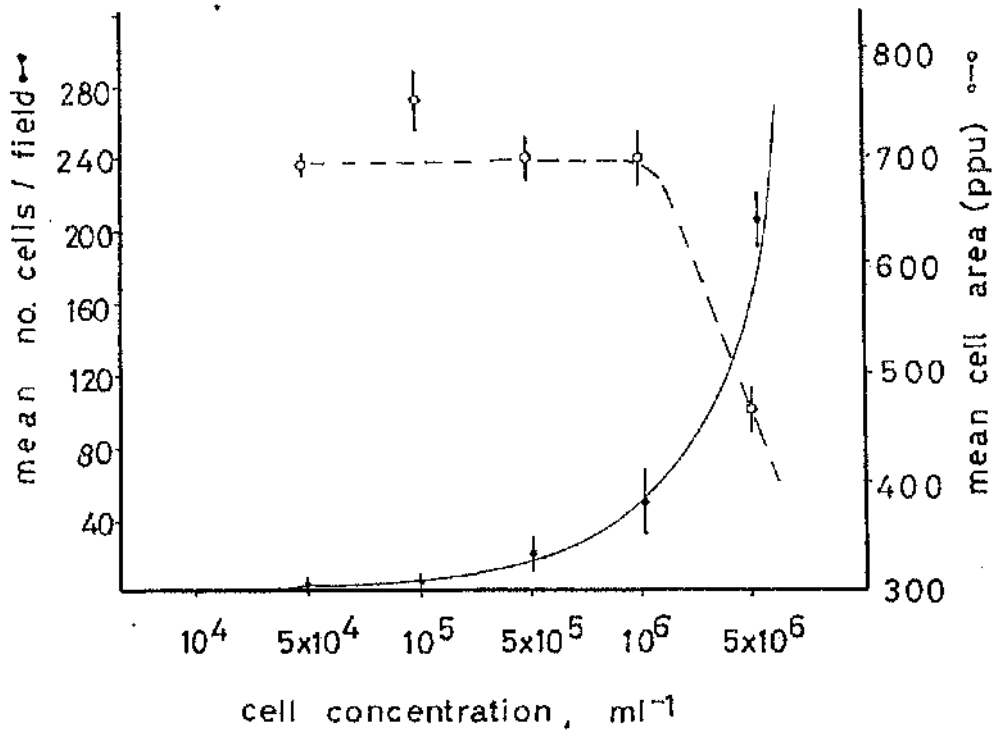
1 field = 2.867×10^5 μm^2

ppu = picture point units, 1 ppu = 0.574 μm^2

Figure 3.

Peritoneal exudate cells were incubated for thirty minutes at 37'C in multiwell tissue culture plates containing FCS-coated glass coverslips. The coverslips were washed in PBS, fixed in formol saline and stained with coomassie blue. The numbers of cells attached and the average spread area were measured using an image analyzing computer. The values shown are mean and standard deviations for 10 readings from each of duplicate preparations.

Figure 3. Variation in number and average spread area of adherent cells with concentration of cell suspension.



1 field = 2.867×10^5 μm^2

ppu = picture point units, 1 ppu = 0.574 μm^2

$$\text{number of contacts} = \frac{n(n-1)\pi d^2}{2A}$$

where n = no. of particles per field (cells)

d = diameter of particles, ie. assuming they are round

A = total area of field

(Abercrombie and Heaysman, 1954).

As shown in table 2 there is a large increase in the number of cell-cell contacts at higher cell suspension concentrations, an average of one contact per five cells at a suspension concentration of one million per ml where no decrease in average area is seen and two contacts per five cells at five million per ml where a substantial reduction is seen. To prevent this phenomenon from interfering in subsequent assays, cell suspensions of $1x10^6$ per ml were used, allowing a large number of cells to attach without any apparent diminution of spreading.

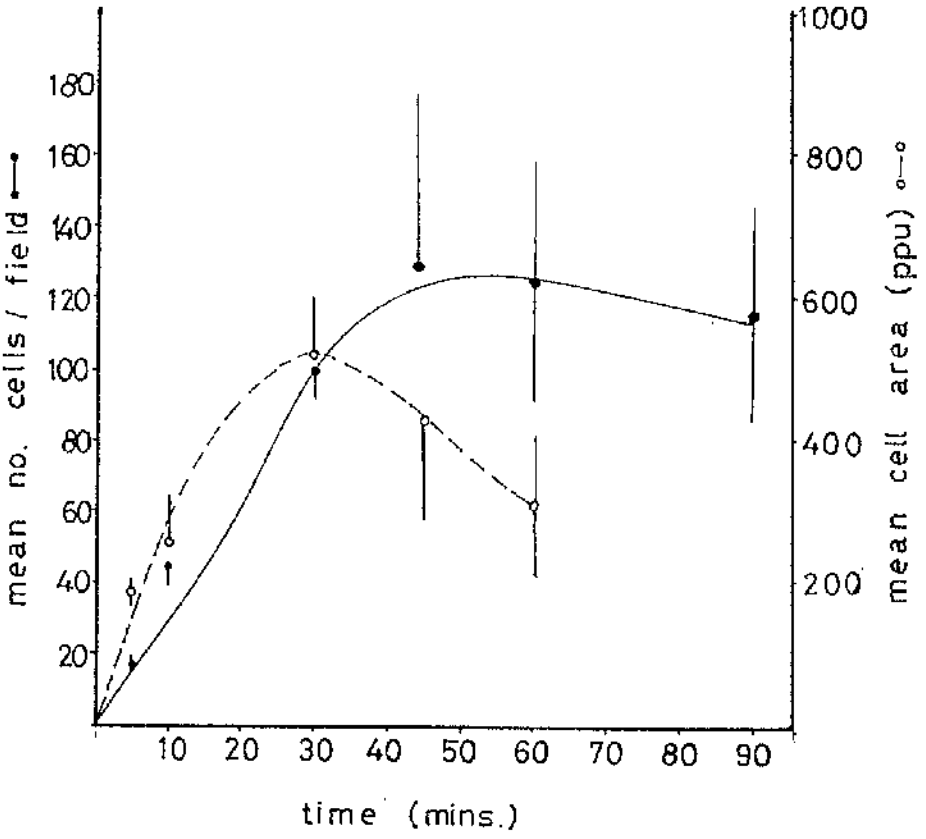
To investigate the effect of incubation time on cell adhesion the number and spread area of cells from a $1x10^6$ cells per ml suspension adhering to FCS coated coverslips at 37°C was monitored over a 90 minute period. From the graph shown in figure 4 it can be seen that as the incubation time increases beyond 30 minutes, the average area of the cells decreases concomitantly with the increase in the number of cells per field. This may again be due to contact inhibition of spreading and for this reason a 30 minute incubation time was used in all further experiments.

To summarize, the optimal conditions for these experiments was found to be a 30 minute incubation of a $1x10^6$ cells ml^{-1}

Figure 4.

One million peritoneal exudate cells were incubated for the times shown at 37'C in multiwell tissue culture plates containing FCS-coated coverslips. The coverslips were washed in PBS, fixed in formol saline and stained with coomassie blue. The numbers of cells attached and their spread area were measured using an image analyzing computer. Values shown are mean and standard deviations from 10 readings from each of duplicate preparations.

Figure 4. Variation in number and average spread area of adherent cells with length of incubation.



1 field = 2.867×10^5 μm^2

ppu = picture point units, 1 ppu = 0.574 μm^2

suspension at 37°C.

Adhesion to syngeneic and allogeneic H-2D antigen fragments.

The adhesion of cells from a variety of mouse strains to H-2D fragments of the b, d and k haplotypes was assayed. In the first instance only the numbers of cells adhering was considered and the results from several of these assays are presented in table 3 and figure 5. To aid clarity and allow comparison between experiments, the number of cells adhering to the syngeneic H-2 antigen is given as 100 in each case, and the number adhering to allogeneic and control preparations as a percentage of this. Absolute values for syngeneic combinations are also given. In every assay performed, a highly significantly larger number of cells attached to syngeneic than to allogeneic fragments, allogeneic attachments numbering one half to two thirds that of syngeneic. The counts on the two allogeneic fragments was significantly different for two of the four strains used : for Balb/b PEC adhesion to H-2D^{k} was greater than to D^{d} fragments and both these greater than to FCS control. Similarly for B10.HTT where the order was H-2D^{b} > D^{k} > FCS. With the other two strains used, B10.BR and B10.S(7R), no significant differences either between allogeneic fragments or between allogeneic and control preparations were found.

To investigate whether the nature of adhesion differed between syngeneic and allogeneic combinations two methods were used. Firstly interference reflection microscopy, a technique which shows up areas of closest contact between cell and substrate as dark regions on a light background. This failed to detect any

Table 3.

One million peritoneal exudate cells were incubated for 30 minutes at 37'C in multiwelll tissue culture plates containing coverslips treated either with an H-2D antigen preparation or with FCS. The coverslips were washed in PBS, fixed in formol saline and stained with coomassie blue. The number of cells attached was measured using an image analyzing computer. In the results, syngeneic combinations are given as 100, other combinations as a percentage of syngeneic. The results are mean and standard deviations from 10 readings per preparation and duplicate preparations.

Table 3. Adhesion of syngeneic and allogeneic cells to H-2D antigen coated coverslips.

PEC		Haplotype of H-2D fragment			Control
strain	haplotype	k	d	b	FCS
Balb/b	b	75±15**	66±8**	100±17	57±16**
		71±11**	63±8**	100±17	56±13**
B10.HTT	d	44±16**	100±17	66±12**	29±12**
		55±13**	100±11	60±14**	36±7**
B10.BR	k	100±13	66±10**	58±7**	60±11**
		100±14	54±12**	54±14**	47±4**
B10.S(7R)	d	56±9**	100±8	63±19**	63±15**
		62±14**	100±13	69±18**	61±10**

Values given as mean number of cells per field + std. dev.

* significantly less than syngeneic combination (P<5%)

** highly sig. less than syngeneic combination (P<1%)

t test performed using Cochrane and Cox's modification.

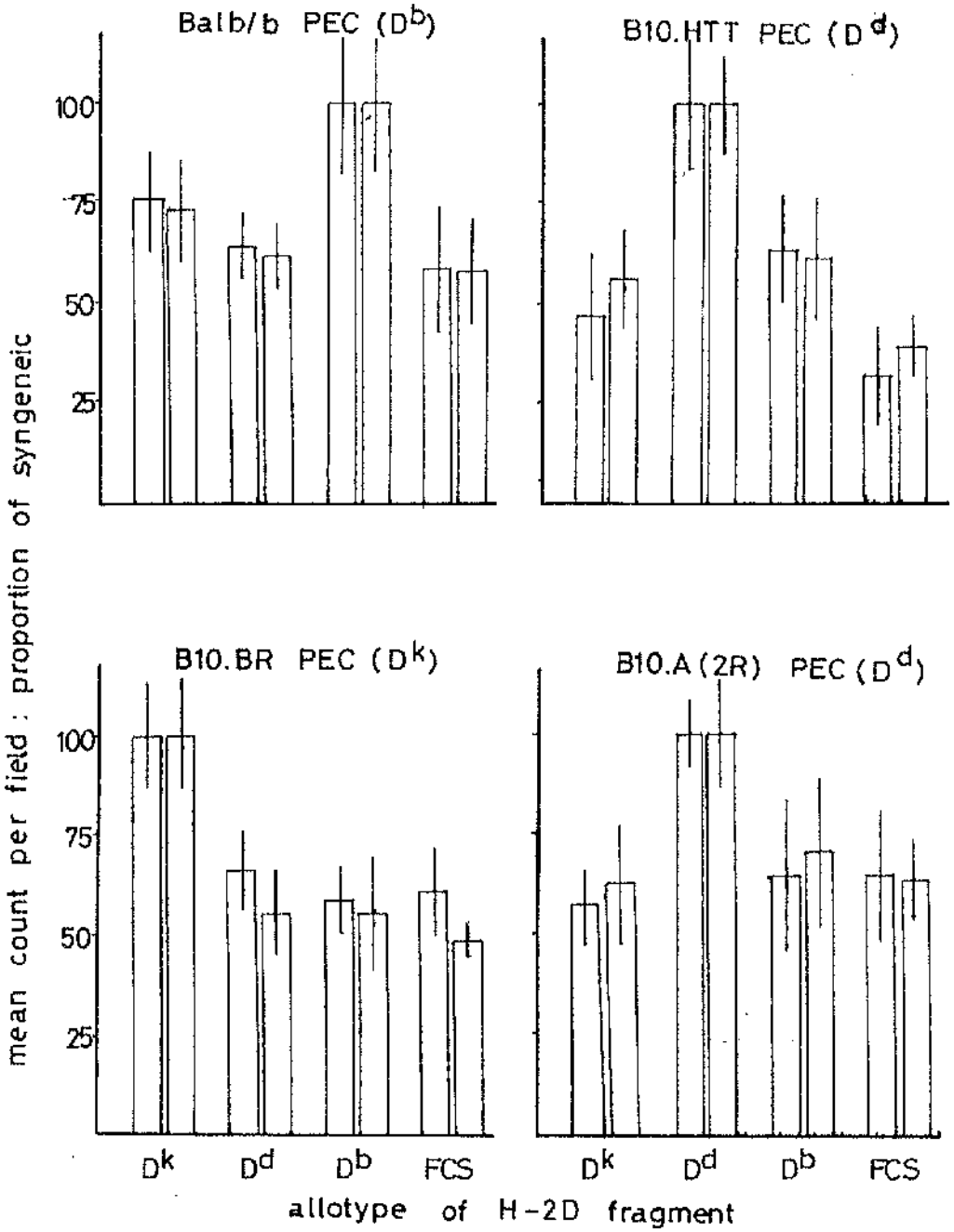
Absolute values for syngeneic combinations:

Balb/b	first experiment	185
	second experiment	175
B10.HTT	first experiment	163
	second experiment	173
B10.BR	first experiment	82
	second experiment	102
B10.S(7R)	first experiment	205
	second experiment	230

Figure 5.

One million peritoneal exudate cells were incubated for 30 minutes at 37'C in multiwell tissue culture plates containing coverslips treated either with an H-2D antigen preparation or with FCS. The coverslips were washed in PBS, fixed in formol saline and stained with coomassie blue. The number of cells attached was measured using an image analyzing computer. In the results, syngeneic combinations are given as 100, other combinations as a percentage of syngeneic. The results are mean and standard deviations from 10 readings per preparation and duplicate preparations.

Figure 5. Variation in mean number of cells per field with different cell-substrate combinations.



differences. Measuring the average spread area of the cells did, however, reveal differences between the two as can be seen in figure 6 and table 4. Again syngeneic values are given as 100. While the total number of cells attached is again greater for syngeneic than allogeneic combinations, the reverse is true for the average spread area of the cells which are highly significantly for allogeneic combinations.

Table 4.

One million peritoneal exudate cells were incubated at 37'C for 30 minutess in multiwell tissue culture plates containing coverlips treated either with an H-2D antigen preparation or with FCS. The coverslips were washed with PBS, fixed in formol saline and stained in coomassie blue. The number and average spread area of attached cells was measured using an image analyzing computer. In the results, syngneic combinations are given as 100 and other combinations as a percentage of this.

Table 4. Cell count and average spread area of cells on syngeneic and allogeneic H-2D coated coverslips.

PEC strain	PEC haplotype	substrate	count (per field)	area (ppu)
B10.BR	k	D^k	100±4	100±5
		D^b	82±7**	126±5**
Balb/b	b	D^k	83±8**	131±4**
		D^b	100±8	100±1

Values are given as mean + standard deviation

1 field = 2.867×10^5 μm^2

ppu = picture point units, 1 ppu = 0.574 μm^2

** highly significant (P<5%).

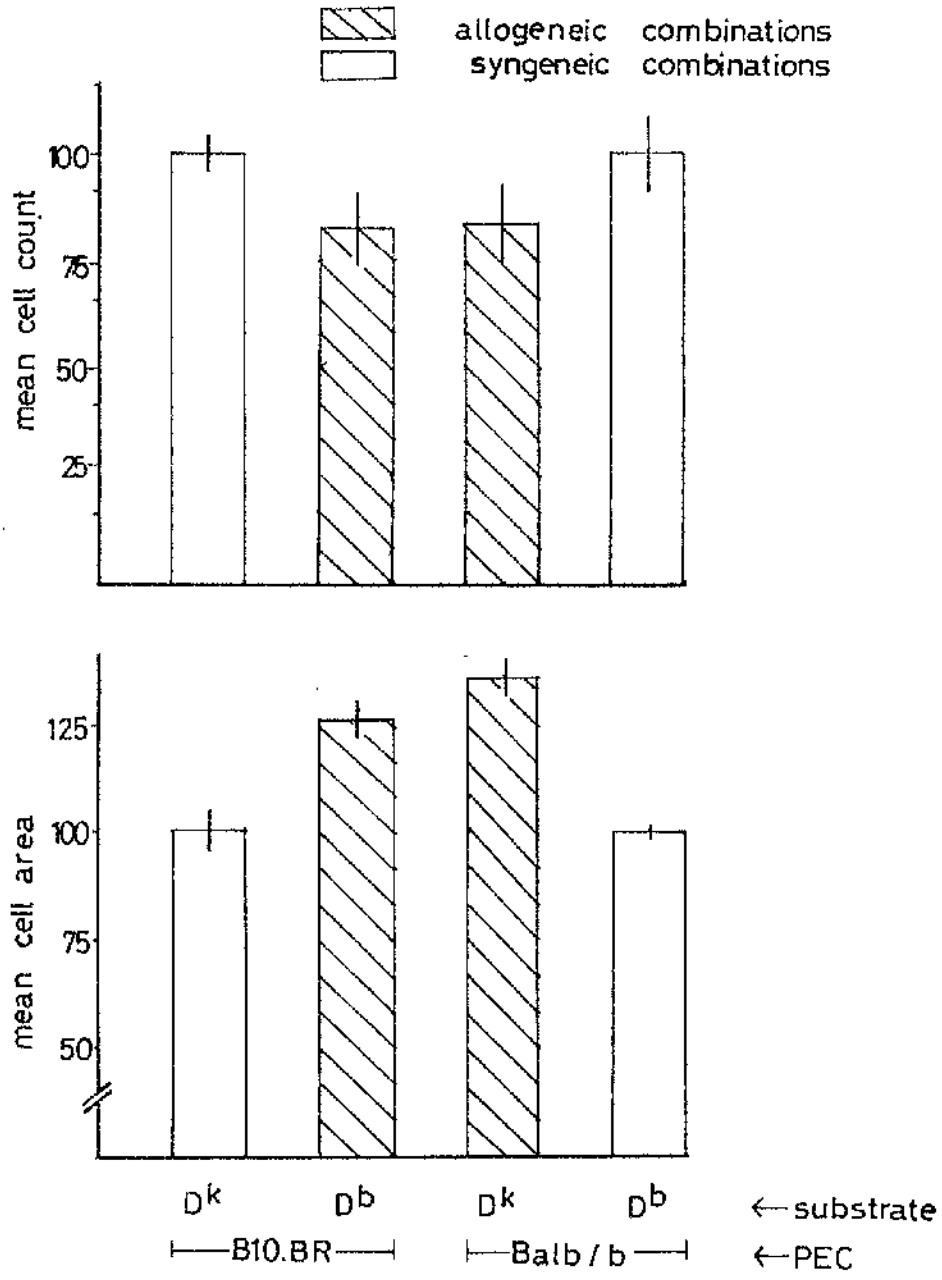
Absolute values for syngeneic combinations:

	count	area
B10.BR	138	395
Balb/b	173	308

Figure 6.

One million peritoneal exudate cells were incubated at 37'C for 30 minutess in multiwell tissue culture plates containing coverlips treated either with an H-2D antigen preparation or with FCS. The coverslips were washed with PBS, fixed in formol saline and stained in coomassie blue. The number and average spread area of attached cells was measured using an image analyzing computer. In the results, syngneic combinations are given as 100 and other combinations as a percentage of this.

Figure 6. Adhesion of syngeneic and allogeneic peritoneal exudate cells to H-2D fragment coated coverslips.



Discussion.

A criticism of this work could be that the cells used, peritoneal exudate cells, were not a homogeneous population but a mixture of cell types, mostly macrophages but with up to 50% small lymphocytes (Hudson and Hay, 1980). This was an adhesion assay, however, and after 30 minutes at 37°C an adherent subpopulation is selected for which contains few lymphocytes. This technique in fact constitutes one of the simplest methods of enhancement of a macrophage population. Methods for the purification of macrophages such as adhesion followed by trypsinization or carbonyl iron ingestion (Mishel and Shiigi, 1980) were not carried out because of their possible perturbing effects on the cell surface.

From the results the main conclusion is that H-2D antigens have a modifying effect on macrophage adhesion and that this is manifest as adhesion in greater numbers to syngeneic than to allogeneic H-2D coated coverslips. This is in agreement with Zeleny et al (1978) and Curtis and de Souza (1975) who demonstrated greater adhesion of syngeneic over allogeneic lymph node cells to peritoneal cell monolayers, but disagrees both with Curtis and Renshaw (1982) who found that syngeneic combinations of lymphocytes and endothelial cell monolayers were less adhesive than allogeneic, and with Haston (1979) who found no differences between the two in experiments using lymphocytes and thymocytes. Bartlett and Edidin (1978) have described a hierarchy of haplotypes where H-2^s strains were most successful and H-2^k strains the least successful fibroblast monolayers in picking up

single fibroblasts from suspension. In the experiments reported in this chapter, no absolute hierarchy was found : for Balb/b the order was b >k >d >FCS and for B10.HTT it was d >b >k > FCS. For the other two strains used, B10.BR and B10.S(7R), syngeneic combinations were most efficient but there were no significant differences between other combinations.

The combination of allogeneic cells and substrates resulted in larger average spread areas per cell than with syngeneic combinations and there is more than one possible reason for this. If the allogeneic interactions are weaker than syngeneic then a large number of them may be required to achieve adhesion. After the thirty minute period of incubation those cells which had not spread sufficiently would be removed on washing of the coverslips whereas with syngeneic combinations the stronger attachments would allow them to remain adherent. This population of less well spread cells would reduce the average spread area of the cells while increasing the total number of cells attached. The difference in the number of cells attached can also be explained by a mechanical argument: the shear stress felt by a cell is proportional to its radius (Visser, 1976) and, since the allogeneic cells spread more than the syngeneic, it is possible that they are detached when the coverslips are washed. A more simple explanation is that, since the number of cells attaching to syngeneic H-2D coated coverslips is much larger than to control FCS coated coverslips, contact inhibition of spreading may be affecting the results, much as in figure 3. This possibility could be investigated simply by reducing the

concentration of the cell suspension used. Longer and shorter incubation periods may also yield interesting results.

In retrospect the choice of FCS as a control may have been an unfortunate one because it may contain factors inhibitory to cell adhesion (Curtis et al, in press). This raises the possibility that, rather than syngeneic combinations enhancing adhesion, allogeneic combinations are being inhibited since the adhesion to allogeneic fragments was often found not to be significantly different to adhesion to FCS. This possibility could be investigated by repeating the experiments using as a control a protein known to enhance the adhesion of cells to glass substrates, for example fibronectin (Virtanen et al, 1982).

The most puzzling and important questions to come from these experiments concern the nature of the involvement of the H-2D locus in adhesion. The results above suggest that the haplotype of both the cell and fragment are important but they cannot be explained by a simple like-like interaction between H-2D molecules since expression of H-2D is ubiquitous and not all cells are equally adhesive. The use of mouse strains identical only at the H-2D locus may show whether this locus or another part of the H-2 complex is important in, for example the coding of a receptor for H-2D.

CHAPTER 4.

ATTACHMENT OF SOLUBLE Ia (CLASS II) ANTIGENS TO PERITONEAL EXUDATE CELLS AND LYMPHOCYTES.

Introduction.

Two subpopulations of macrophages have been identified on the basis of Ia (class II) antigen expression: Ia^+ macrophages are more common in the spleen (Cowing et al, 1979), thymus (Beller and Unanue, 1980) and liver (Richman et al, 1979) than Ia^- which predominate in peritoneal exudates (Cowing et al, 1979). There is a very close correlation between Ia positivity of a macrophage population and its ability to cooperate in immune responses, for example in mixed lymphocyte reactions (MLR) (Schwartz et al, 1976), antigen or mitogen-induced proliferation (Farr et al, 1979a ; Habu et al, 1979) and lymphokine production (Farr et al, 1979b). The relationship between the two subpopulations is not entirely clear: in vivo, immunological stimuli are more likely to recruit Ia^+ macrophages into the peritoneum than switch on expression in the resident cells (Beller et al, 1980) although in vitro interconversion both from Ia^+ to Ia^- (Cowing et al, 1979) and from Ia^- to Ia^+ has been described (Steeg et al, 1979). The latter induction of Ia expression, was acheived using a lymphokine preparation from con A stimulated spleen cells. Culture of peritoneal macrophages for seven days in this lymphokine preparation increased the percentage of cells expressing Ia from less than 10 to up to 80.

It would be of great interest to know whether the expression of Ia antigens on the surface of viable macrophages alone confers ability on these cells to cooperate in immune responses or whether other concomitant changes are also necessary.

The acquisition of Ia antigens by uptake from medium has been described for T helper cells (Delovitch et al, 1982) and alloreactive T cells (Lorber et al, 1982) which adsorb nonself Ia antigens. It was the intention of this work to find out if an Ia^- population of macrophages could absorb Ia antigens in a similar manner to T cells and, if possible, to test whether this population could cooperate in the immune response.

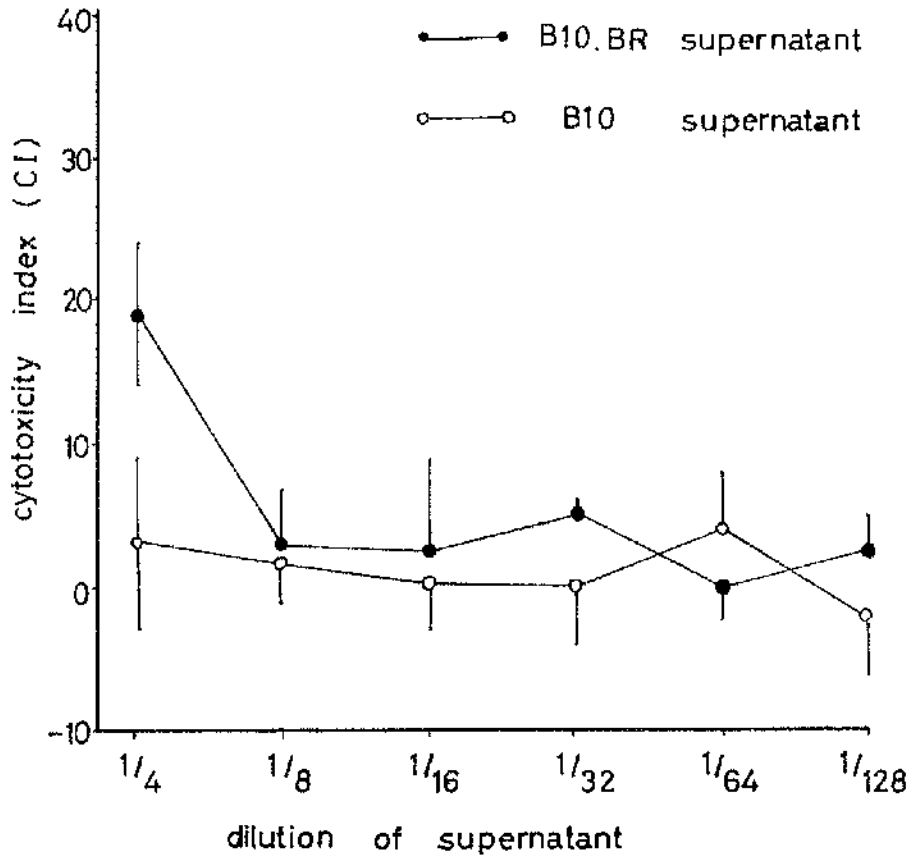
Incubation of PEC with con A supernatant.

Using the method described by Steeg et al (1980), lymphokine (LK) preparations were made from B10 and B10.BR strains. Steeg et al (1980) incubated cells with LK for seven days before assaying for Ia expression and concluded that adsorption of molecules was not responsible for the increase. In all likelihood any Ia antigens present in the supernatants would have been degraded in that period, so to determine whether absorbed Ia was detectable on the PEC surface, overnight incubations of cells with LK preparations were attempted and are reported here. Figure 7 shows the results of the initial experiment using B10 PEC, both B10($H\text{-}2^b$) and B10.BR($H\text{-}2^k$) LK preparations and anti-Ia^k antiserum in the complement-dependent cytotoxicity assay. Guinea-pig serum, the complement source, was used at 1/10, 1/100 and 1/1000 dilutions. The results shown are for the 1/100 dilution which gave appreciable lysis in experimental, but not control cultures. It can be seen from figure 7 that Ia expression detectable where the cells were incubated in the 1/4 dilution of B10.BR LK and in no other preparation.Thus it appears that Ia antigens are adsorbed from the supernatant in an antigenically recognizable form and

Figure 7.

Twenty thousand peritoneal exudate cells were added to wells in flat-bottomed microtitre plates and incubated for 30 minutes at 37'C. Non-adherent cells were washed off and adherent cells treated either with RPMI containing Ia antigen preparation or RPMI alone. After overnight incubation the cells were thoroughly washed with PBS before incubation in 1/200-diluted anti-Ia antiserum for 60 minutes at 4'C. The cells were then thoroughly washed before the addition of 1/100-diluted guinea-pig serum. After one hours' incubation at 37'C, the complement was removed and the degree of lysis determinedby trypan blue exclusion. Results are given as mean asnd standard deviations of triplicate cultures.

Figure 7. Comparison of B10 and B10.BR con A supernatant stimulation of Ia antigen expression by PEC after overnight incubation.



Significant difference only at 1/4 dilution.

survive at least for several hours. In a second, more extensive experiment, B10 and B10.BR PEC were reacted with syngeneic and allogeneic preparations and assayed after both overnight and seven day incubations. These results are presented in figure 8. Overnight incubation of both types of PEC with B10.BR supernatant results in significant expression of Ia^k antigens at 1/4 dilution whereas at 7 days only B10.BR cells, treated with both LK types, express Ia. This suggests that exogenous Ia^k antigens are absorbed onto both syngeneic and allogeneic cells and that after seven days in culture the expression of Ia is endogeous. The expression of Ia^b was not assayed because of the unavaiability of an anti-Ia^b antiserum and the expense of the available monoclonal reagents.

At this point it was decided, for several reasons, to attempt to make a purified Ia antigen preparation. Firstly, the preparations used previously were unpurified cell supernatants and the possibility of some, perhaps more complicated, reasons for the increase in Ia positivity could not be overlooked eg. con A induction of expression. Furthermore the supernatants were active only at very high concentrations (1/4) and caused recognizable expression of Ia antigens in only a small proportion of cells (25% at best).

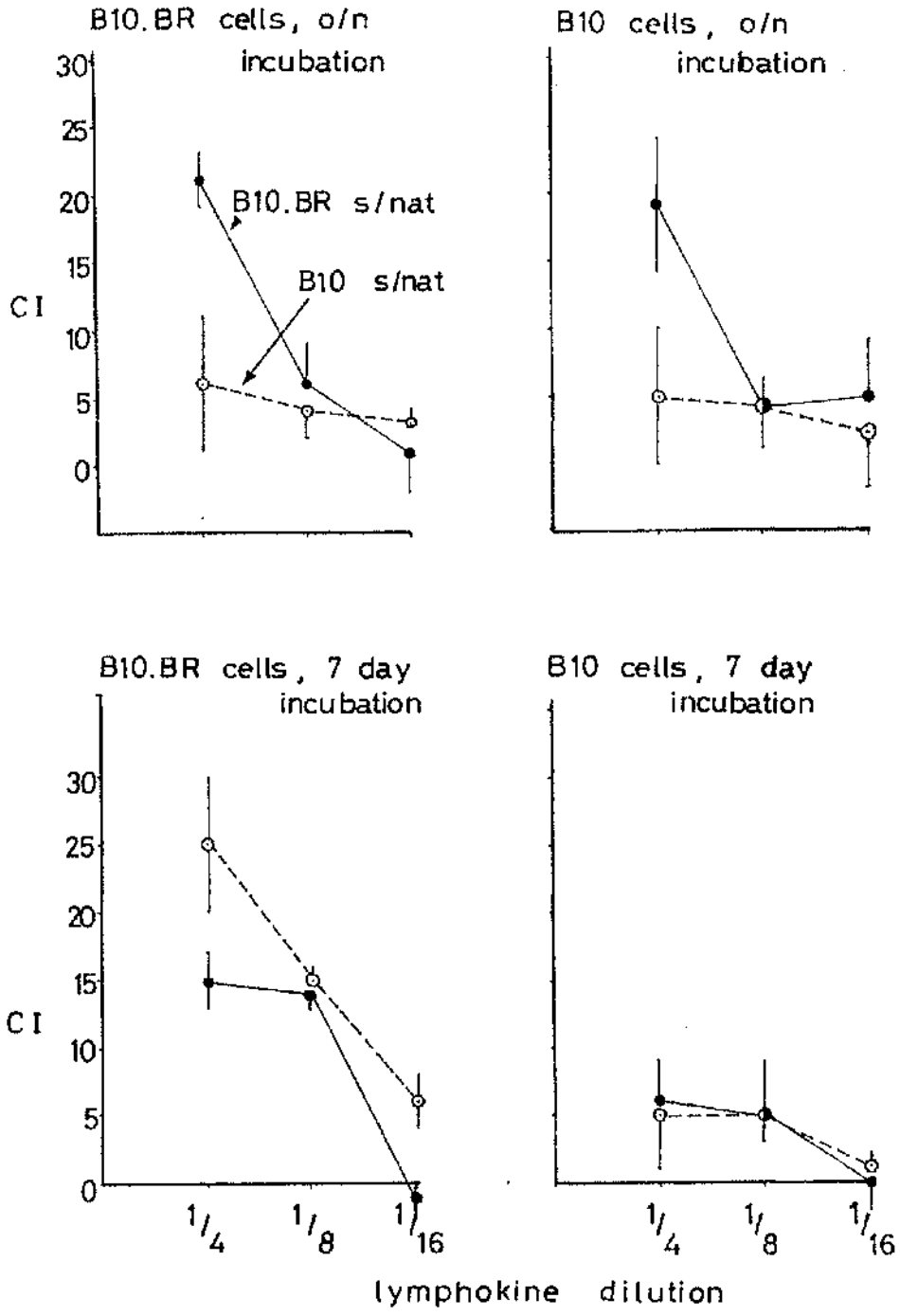
Ia antigen preparation.

It was thought that one possible method for increasing the yield of Ia antigens may be to employ a B cell mitogen, such as lipopolysaccharide, in place of concanavlin A, a T cell mitogen. The method of Nagy et al (1982) was used but, rather than

Figure 8.

Twenty thousand peritoneal exudate cells were added to wells in flat-bottomed microtitre plates and incubated for 30 minutes at 37'C. Non-adherent cells were washed off and adherent cells treated either with RPMI containing Ia antigen preparation or RPMI alone. After overnight or seven day incubation the cells were thoroughly washed with PBS before incubation in 1/200-diluted anti-Ia antiserum for 60 minutes at 4'C. The cells were then thoroughly washed before the addition of 1/100-diluted guinea-pig serum. After one hours' incubation at 37'C, the complement was removed and the degree of lysis determined by trypan blue exclusion. Results are given as mean asnd standard deviations of triplicate cultures.

Figure 8. Ia antigen expression in PEC after overnight and seven day incubation in lymphokine preparation.

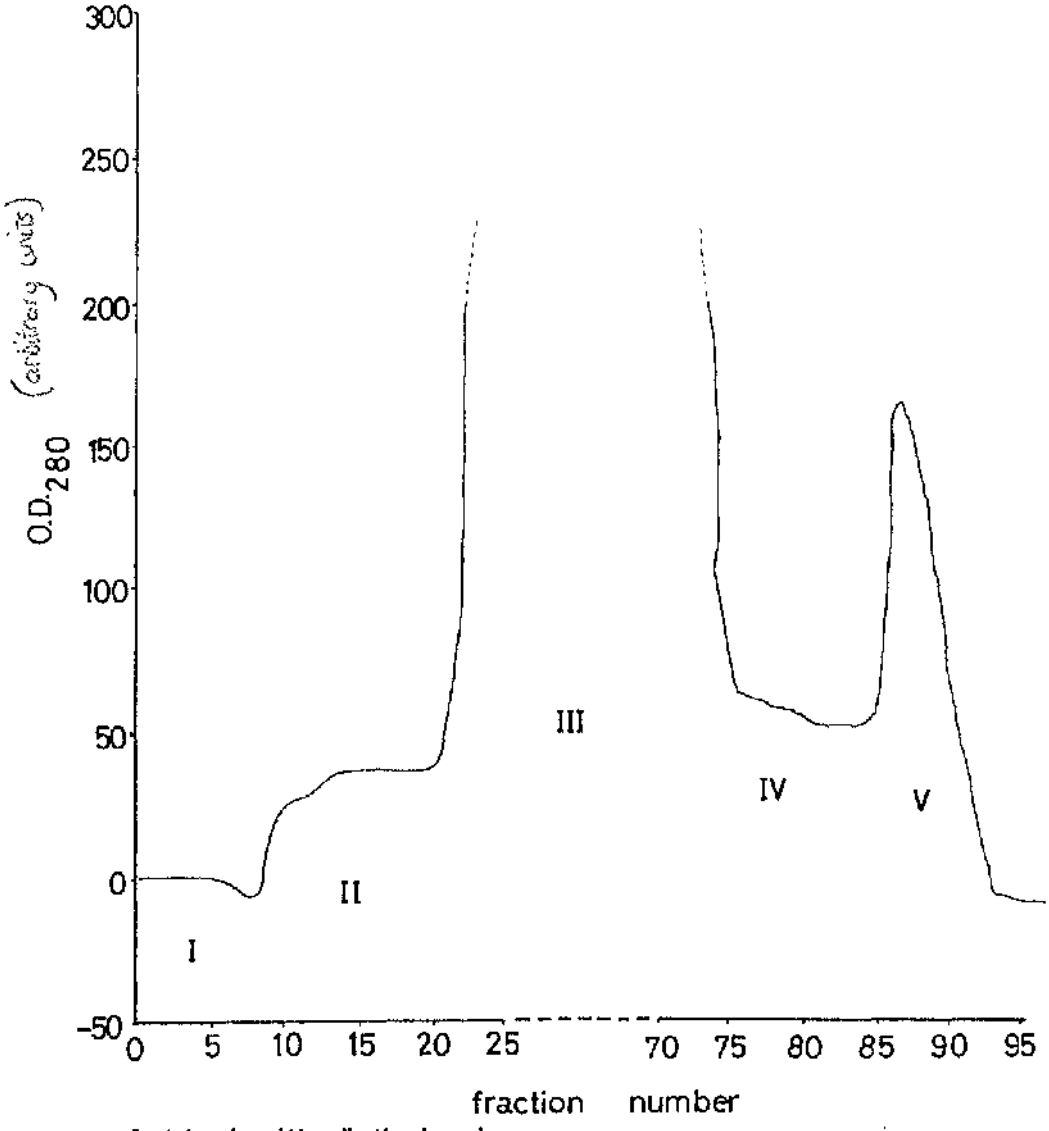


concentrating with Amicon filters, the supernatant was applied to a monoclonal anti-Ia^k antibody column as described in materials and *methods*, *section* 8. The OD_{280} readings of fractions eluted from the column are shown in figure 9. Readings were taken every 0.1ml and eluate collected in 0.5ml fractions. The small but distinct peak eluted with diethylamine was collected and diethylamine removed under vacuum. To check the preparation, 10µl of the sample (from fraction 87) was run on an SDS-PAGE gel in duplicate along with a series of molecular weight standards. The proteins were blotted onto nitrocellulose paper, one half stained in amido black to ensure that the transfer had been successful (figure 10a) and the other treated to determine whether Ia antigens were present in the peak. This was done by firstly incubating the paper with 1% BSA overnight to block non-specific protein binding sites, then reacting it with 1/50-diluted anti-Ia^k antiserum at room temperature. The antiserum was washed off before the application of ^{125}I-protein A for six hours at room temperature and finally exposure to X-ray film for three days. The result of this procedure can be seen in figure 10b : one large spot in the track of the Ia antigen preparation, nothing in the molecular weight standard track. On the paper stained with amido black the molecular weight standards are clearly visible, indicating successful transfer. Nothing can be seen on the other track as the limit of resolution of amido black is relatively low (in the microgram range). Taken together, these results strongly suggest that Ia antigens are being purified by this method and detected in the blotting assay. Silver staining of an SDS-PAGE gel (figure 11) reveals three bands in the 60-70 kD

Figure 9.

An absorbant column was prepared by conjugating an anti-Ia monoclonal antibody to cyanogen bromide-activated Sepharose 4B. The column was equilibrated with diethylamine and PBS before culture supernatants were applied. After re-equilibrating the column with PBS, diethylamine (0.005M, pH 11.5) was washed through to remove specifically bound proteins. Fractions from this peak were pooled and diethylamine removed under vacuum.

Figure 9. Elution through monoclonal anti-Ia^k column.



I blank with diethylamine
II equilibration with PBS
III Ia antigen preparation through column
IV equilibration with PBS
V elution with diethylamine

Figure 10.

Duplicate 50µl samples of material eluted from the monoclonal antibody column (see figure 9) were run, along with molecular weight standards, on an SDS-polyacrylamide gradient gel (5-12%). Samples were not boiled. After the gel was run the proteins were transferred onto nitrocellulose paper. The paper was halved, one half stained with amido black to check the success of transfer and the other treated with 1/50-diluted anti-Iaantibody for 30 minutes. After washing the paper, it was treated for a further six hours at room temperature with a solution containing radioiodinated protein A. The paper was thoroughly washed and exposed to X-ray film for three days. On developing the film, one large spot could be seen.

Figure 10. Immunoblot of Ia antigen preparation.

(a) Amido black staining of nitrocellulose paper

(b) Developed autoradiograph.

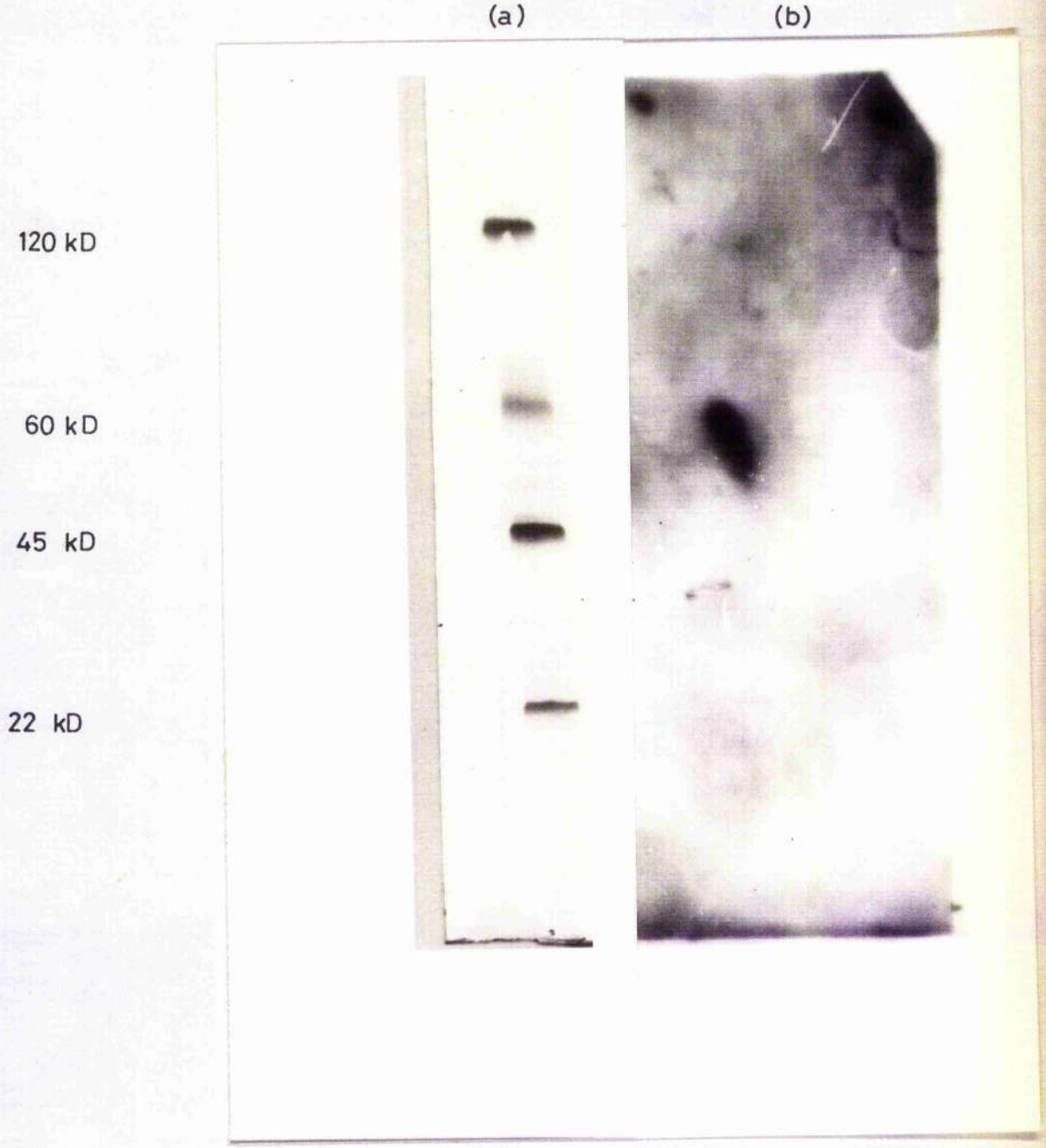
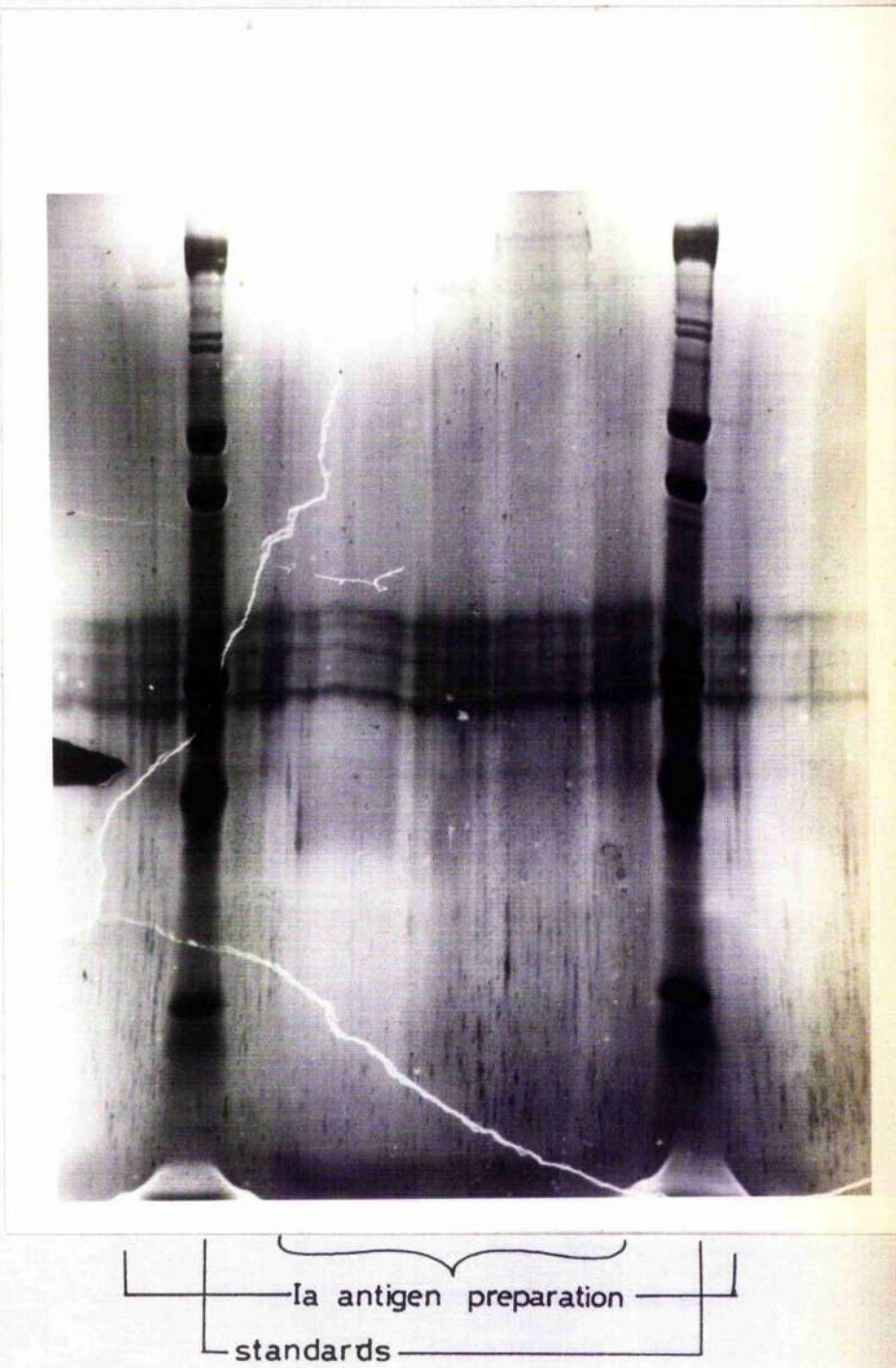


Figure 11.

50 µl samples of material eluted from thre monoclonal antibody column (see figure 9) were run alongside molecular weight standards on an SDS-polyacrylamide gradient gel (5-12%) and the gel silver stained.

Figure 11. Silver staining of Ia antigen preparation.



region, identical in column fractions 85, 87 and 89. It appears that, rather than splitting into constituent α and β chains (28 and 33 kD respectively) the antigens have remained as a dimer.

Attachment of soluble Ia antigens to peritoneal exudate cells.

50µl samples of ^{125}I-conjugated Ia^k antigen preparation were incubated with 30 minute-adherent PEC for 30 minutes, extensively washed and counted in a gamma counter. The results are shown in table 5: 65-100% of total activity was recovered, indicating substantial attachment of Ia^k antigens to both syngeneic and allogeneic cells. With this knowledge it was decided to test what effect, if any, these fragments would have on in vitro immune responses.

Effect of Ia antigens on mixed lymphocyte reactions.

Initially it was hoped to study the effect of Ia antigens on T cell proliferation assays using peritoneal exudate macrophages to present TNP-BGG to immune lymph node cells. Unfortunately these assays did not yield consistent results and they are not presented here. Instead, mixed lymphocyte reactions were performed which, although technically easier, are more difficult to interpret. Figure 12 shows the results from two of these assays. Using H-2^b lymphocytes as stimulators (B10 and Balb/b) a highly significant reduction in uptake of tritiated thymidine was observed where the Ia^k antigens were included. Using H-2^k strains as stimulators, in one case (B10.BR) the response is enhanced, while in the other no increase is seen, possibly because there is maximal activation in the control which would suppress the appearance of any effect. The inclusion of Ia

Table 5.

One million peritoneal exudate cells were incubated for 30 minutes at 37'C in multiwell tissue culture plates containing glass coverslips. After the removal of nonadherent cells, PBS containing 500µl of iodinated Ia antigen was added and the plates were incubated for a further 30 minutes at 37'C. Coverslips were thoroughly rinsed and the amount of labelled protein bound determined. Results shown are mean and standard deviations of triplicate cultures.

Table 5. Attachment of ^{125}I-Ia^k antigen preparation to PEC.

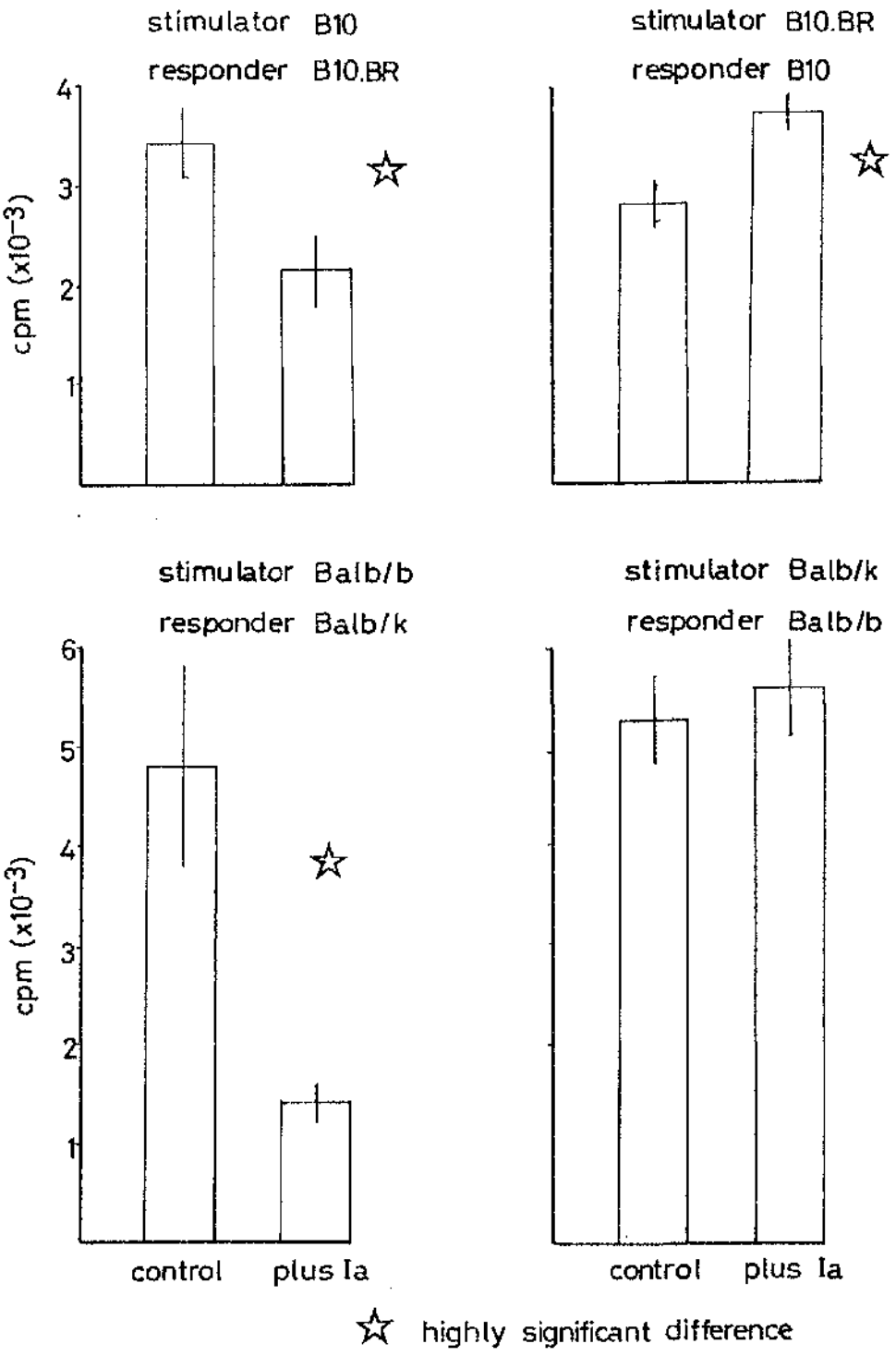
Mouse strain	c p m
B10.BR	4517± 260
B10.A	4903±1693
B10	3182±1614
B10.D2	5309±1355
B10.HTT	3577±746
50µl sample of preparation	4864

cpm = scintillation counts per minute

Figure 12.

Four hundred thousand stimulating cells, previously treated with mitomycin C were mixed with two hundred thousand responder cells in a final volume of 1 ml and cultured for 96 hours at 37'C. 1uCi of tritiated thymidine was added for the final four hours of culture before the cells were harvested onto glass-fibre filters and measured in a scintillation counter. Results shown are mean and standard deviations of triplicate cultures.

Figure 12. Uptake of tritiated thymidine in mixed lymphoctye reactions with and without the addition of Ia antigen preparation.



antigens in experiments using stimulators and responders over a range of concentrations could prove or disprove this.

Discussion.

Possibly the most important aspect of the work presented in this chapter is the successful purification of Ia^k antigens from the supernatant of LPS-activated spleen cells. It is unfortunate that another Ia preparation, for example from B10 mice was not made. It would have been useful both as a control in the Ia purification and for a parallel series of mixed leucocyte reactions. In the guinea-pig Schwartz et al (1977) described two types of Ia antigen with respect to electrophoretic properties, one of which ran as two bands, at 25 and 33 kD, the other as a single band at 65 kD under nonreducing conditions. With the addition of 2-mercaptoethanol all antigens ran as two bands, suggesting that the second class had interchain disulphide bond(s) although this may have been the result of the slightly oxidizing conditions used in solubilization (Cook et al, 1978). The same patttern is seen in the preparation described here, where samples were not boiled, and bands are found between 60 and 70 kD. Cullen et al (1979) have reported that the α and β chains of both I-A and I-E antigens run as a complex in the presence of 2-mercaptoethanol if the samples are not boiled first. Since extensive denaturation is thought to occur during electrophoresis it is surprising that blotted antigens interact with antibody raised to native protein, a feature of all immune blotting. Either denaturation is not as severe as previously thought, which is unlikely, or some renaturation occurs, perhaps

driven by the association of hydrophobic residues.

It has been demonstrated by Steeg et al (1980) that the induction of endogenous Ia antigen expression in macrophages enhances the stimulatory capacity of these cells in mixed lymphocyte reactions. Interpretation of the experiments reported here, where soluble Ia antigens are added to splenic mononuclear cells is more difficult. While it is known that the predominant stimulating determinants are encoded in the I region (Bach et al, 1972) and that only antisera against the stimulatory Ia antigen successfully inhibit the reaction (Meo et al, 1978), no reaction occurs between populations of purified lymphocytes. In both murine and human studies a monocyte/macrophage cell is also required which can be syngeneic or allogeneic with the responder lymphocyte population (Twoney et al, 1970, Mann and Abelson, 1980). The simplest explanation of this is that the adherent cells fulfill the T cell requirement for IL 1, a possibility that could be checked by substituting macrophages with a purified IL 1 preparation.

Nagy et al (1976) reported that responder T cells activated in MLR have alloantigen derived from stimulators on their surface. In this chapter the attachment of labelled Ia^k antigens to lymphocytes has been demonstrated and, where allogeneic to responder and syngeneic to stimulator cells, shown to enhance stimulation of B10.BR but not Balb/k responders (see figure 12). One experiment which should be done is the direct treatment of an H-2 responder population with the Ia antigen preparation to determine whether the adsorption of stimulator Ia is sufficient

to induce activation. In both experiments where the Ia antigens were allogeneic to stimulator and syngeneic to responders, the addition caused a substantial decrease in activation. Assuming that there is direct interaction between the stimulating and responding populations, rather than mediation by macrophges, these results may be explained in two ways: they may be due to competitive inhibition of the receptor on the responder cells or, by virtue of adsorption of responder Ia antigen, the stimulators not being recognized as nonself. The use of a 'third party' Ia fragment, allogeneic to both stimulator and responder would perhaps distinguish between these two possibilities: inhibition would still occur if the former, but not the latter were correct.

CHAPTER 5.

REACTIVE OXYGEN INTERMEDIATES IN LYMPHOCYTE ACTVATION.

Introduction.

In the early 1970's Novogrodsky and Katchalski (1972 and 1973) demonstrated the *in vitro* transformation of lymphocytes using oxidizing agents such as neuraminidase plus galactose oxidase and sodium periodate.

The superoxide anion, known to be a moderately strong reducing agent and a weak oxidizing agent in aqueous solutions is produced in many biological systems and can be demonstrated extracellularly. It is, by these criteria, a potential candidate for a lymphocyte transforming agent of some biological significance. Furthermore, there is strong circumstantial evidence for the involvement of other reactive oxygen species in lymphocyte activation (see introduction). The work presented in this chapter is an attempt to show, by direct addition of superoxide rather than inhibition of it, whether or not any transformation was detectable.

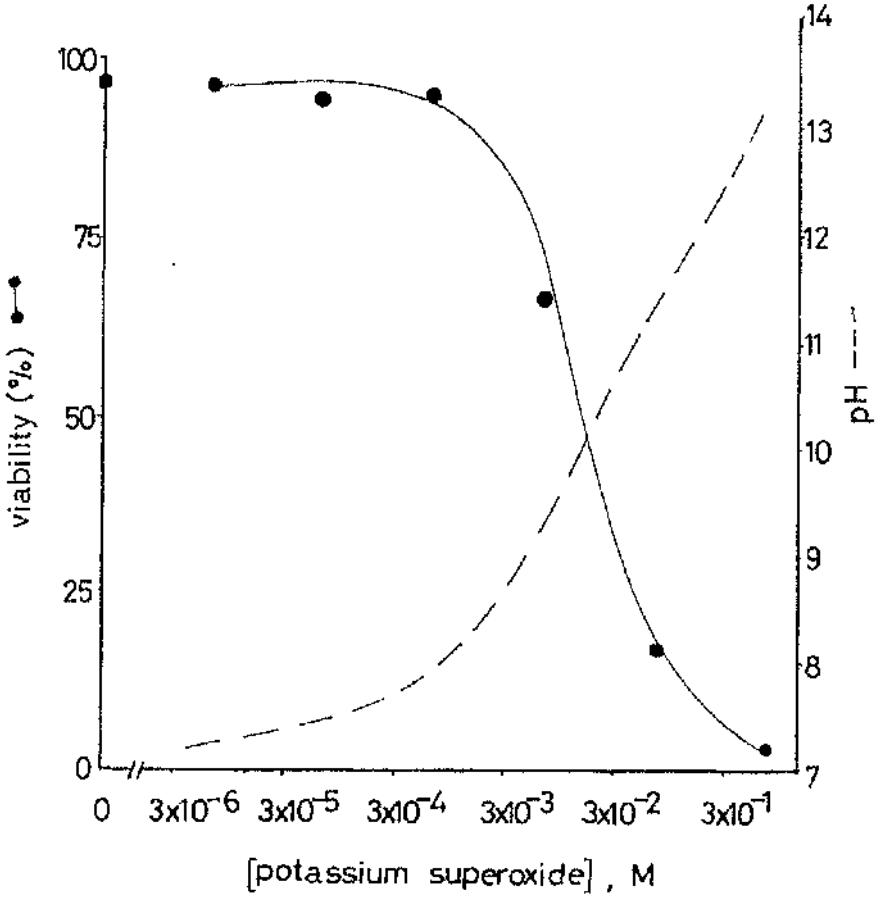
Effect of potassium superoxide on cell viability.

Figure 13 shows the viability of PBM, as determined by trypan blue exclusion, after their incubation for one hour at 37°C over a range of concentrations of potassium superoxide. Also shown is the pH of solutions of potassium superoxide at the same concentrations. Due to its very short half life (a few seconds at millimolar concentrations), solutions of superoxide were not made up until immediately before use. At high concentrations (3×10^{-2} M and higher) the viability was virtually nil and this was attributed to the high pH of the solution: an experiment where

Figure 13.

Human peripheral blood mononuclear cells were exposed for one hour at 37'C to a range of concentrations of potassium superoxide, after which time their viability was determined by trypan blue exclusion. The pH of solutions at the concentrations of potassium superoxide used is also shown and the results are mean and standard deviations of triplicate cultures.

Figure 13. pH of potassium superoxide solutions and their effect on PBM viability.



PBM were incubated with PBS over the same pH range, the increase in pH being brought about using 5N sodium hydroxide in place of potassium superoxide, gave similar results (not shown). At concentrations of $3x10^{-4}$ M and below, viability is equal to that of control and, in all subsequent experiments $3x10^{-4}$ M potassium superoxide was used.

Potassium superoxide as a mitogen.

The uptake of tritiated thymidine by PBM exposed to several conventional mitogens, at optimal concentrations and to potassium superoxide at nontoxic concentrations is shown in figure 14. All, apart from O_2^- were present in the medium for the entire 72 hours of culture. The results suggest that superoxide anions, or a species derived from them, may be involved in lymphocyte transformation.

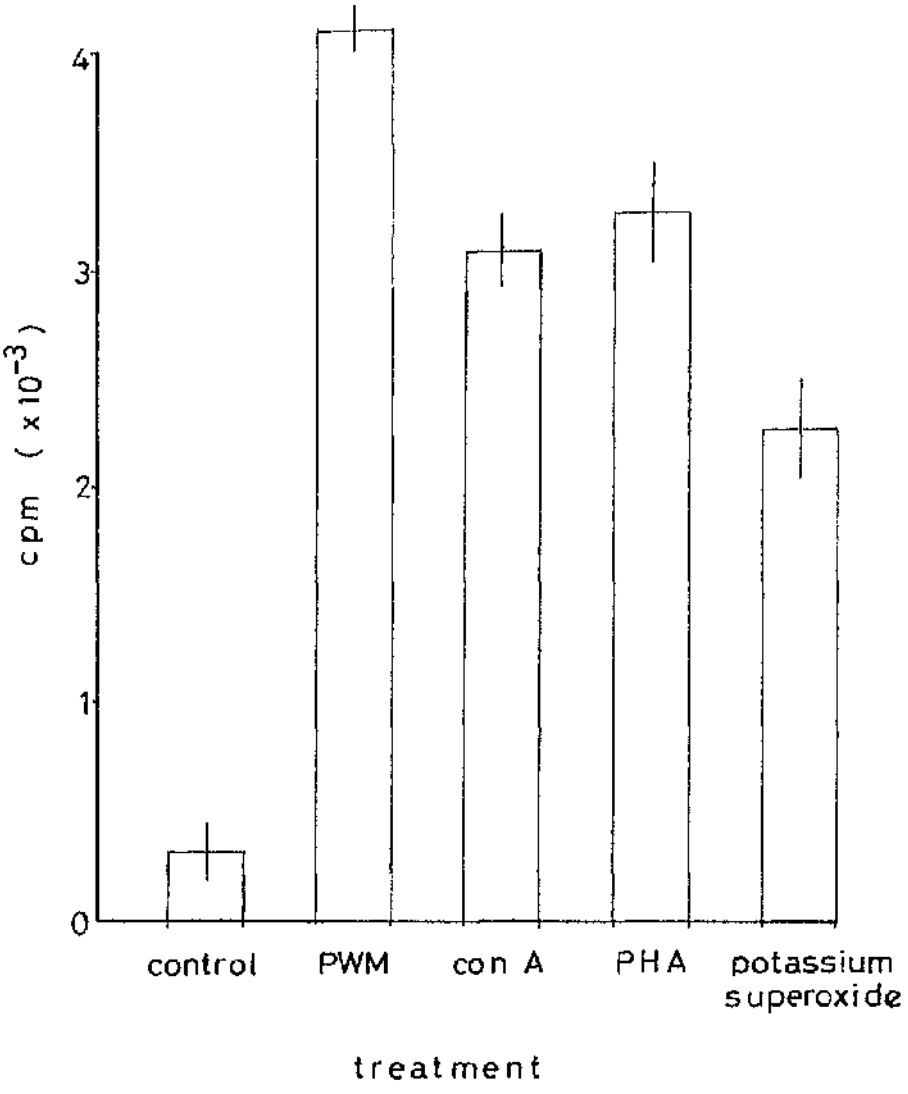
The effect of zymosan-A on uptake of tritiated thymidine by PBM.

The purpose of this experiment was to determine whether stimulation of the monocyte component of the PBM to produce O_2^- would have the same mitogenic effect as the potassium superoxide solution. Several agents are available which induce a respiratory burst in monocytes and of these zymosan-A was chosen, for two reasons: firstly, it has been reported that zymosan-A can induce superoxide production for up to five days in fresh monocytes (Nakagawa et al, 1982). Secondly, there is no evidence in the literature to suggest that zymosan-A, unlike phorbol myristate acetate (PMA) for example, is directly mitogenic for lymphocytes. To check this, $5x10^6$ nylon wool

Figure 14.

Five million peripheral blood mononuclear cells in 1ml were cultured in RPMI 1640 plus 10% autologous serum either alone or with the optimal concentration of a mitogen, for 96 hours at 37'C. Potassium superoxide-treated cells were exposed to 0.00003 M potassium superoxide for one hour at 37'C, thoroughly washed and cultured as above. At the end of the incubation period the amount of tritiated thymidine incorporated was measured. Results are given as mean and standard devaiations of triplicate cultures.

Figure 14. Stimulation of PBM with exogenously supplied superoxide: comparison with mitogens.



purified T lymphocytes were incubated over 72 hours with zymosan-A and these showed no increase over control in uptake of tritiated thymidine (results not shown). Incubation with the complete PBM population does, however, cause an increase in the uptake of tritiated thymidine to about 50% of the level induced by PWM, as can be seen in figure 15. These results can be interpreted in more than on way. For some mitogens, as for MLR, a requirement for adherent accessory cells has been demonstrated It is possible that it is not the superoxide produced by the monocytes which is responsible for the lymphocyte transformation, but their capacity to act as antigen presenting cells. In order to discriminate between these two possibilities, these experiments were repeated with the addition of superoxide dismutase.

The effect of scavengers of reactive oxygen intermediates.

Superoxide dismutase is a very efficient scaveger of superoxide, catalyzing its decomposition to hydrogen peroxide and water. As shown in figure 16, it reduces the uptake of tritiated thymidine almost to background levels, indicating that superoxide may indeed be responsible for the lymphocyte transformation induced by zymosan-A.

In an effort to establish whether or not hydroxyl radicals were responsible for the lymphocyte transformation, mannitol was added to cultures. Mannitol is included in the large and varied list of compounds which are thought to be hydroxyl radical scavengers. Its use in these experiments had no effect on uptake of tritiated thymidine (results not shown) but the possible involvement of

Figure 15.

Five million peripheral blood mononuclear cells in 1 ml were cultured in RPMI 1640 plus 10% autologous serum for 96 hours at 37'C alone, with PWM or with zymosan A. 1uCi of tritiated thymidine was added during the final four hours of culture after which the cells were harvested onto glass-fibre filters and the amount of tritiated thymidine incorporated measured. Results shown are mean and standard deviations of triplicate cultures.

Figure 15. Stimulation of PBM by addition of zymosan-A.

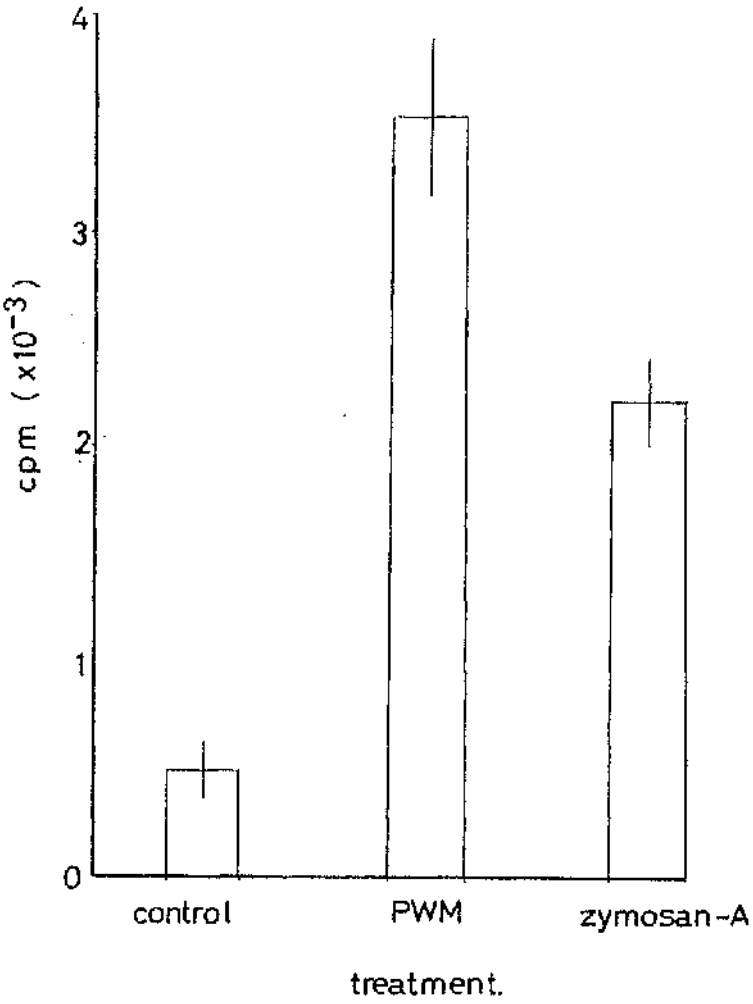
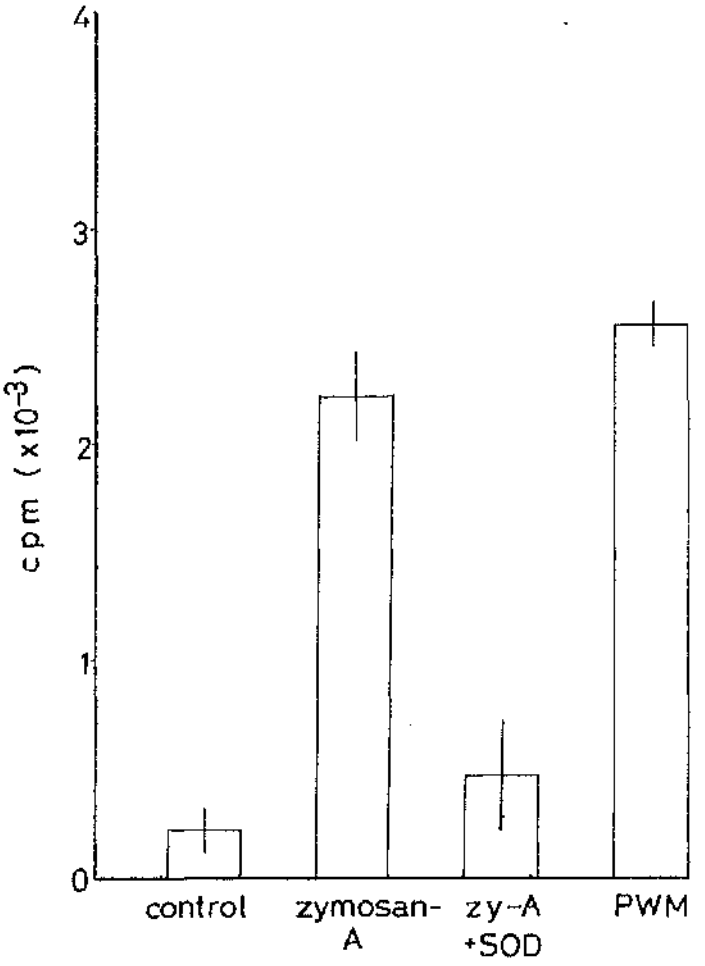


Figure 16.

Five million peripheral blood mononuclear cells in 1 ml were cultured in RPMI 1640 plus 10% autologous serum for 96 hours at 37'C alone, with PWM, with zymosan A or with zymosan A plus superoxide dismutase. 1μCi of tritiated thymidine was added during the final four hours of culture after which the cells were harvested onto glass-fibre filters and the amount of tritiated thymidine incorporated measured. Results shown are mean and standard deviations of triplicate cultures.

Figure 16. The effect of superoxide dismutase (SOD) on stimulation of PBM induced by zymosan-A.



hydroxyl raicals cannot be ruled out, not least because the efficacy of these scavengers is questionable (Bors et al, 1980).

Superoxide treatment and cell adhesion.

Cell adhesion to inert surfaces can be increased by hydroxylation of those surfaces (Curtis et al, 1983). The experiments reported here were done to find out whether superoxide had a comparable effect on cell adhesion. Table 6 shows the change in the average number of particles (single cells or clumps) over a 28 minute incubation period at 37°C for two representative assays. In both cases there are highly significant differences between superoxide and control suspensions, the number of particles in the former decreasing much more rapidly than in controls. Collision efficiencies, which are a quantitative measure of the adhesiveness of a population of cells work out at 28% and 49% for superoxide and 4% and 7% for controls. These are derived from the following equation (see Curtis, 1969):

$$\ln\frac{N_{\infty t}}{N_{\infty 0}} = \frac{-4G\phi t\alpha}{\pi}$$

where G = shear rate/sec
ϕ = volume fraction of cells
$N_{\infty t}$ = number of particles after time t
$N_{\infty 0}$ = number of particles after time 0
π = collision efficiency.

The concentration of O_2^- used in these experiments was crucial: 1mM gave optimal results while increasing the concentration tenfold was toxic to the cells and decreasing fourfold abrogated the effect. Again the solutions had to be used immediately after being made.

Table 6.

One million peripheral blood mononuclear cells in 1 ml of Hanks-HEPES saline, with or without 1mM potassium superoxide solution, were incubated for 30 minutes at 37'C in a Couette viscometer. Samples were removed every 7 minutes and the total number of particles present (single cells or clumps) was determined using an image asnalyzing computer. Results are mean and standard deviation of counts from 10 fields per sample.

Table 6. The effect of potassium superoxide on the adhesion of PBM.

Time (mins)	Expt. 1		Expt. 2		(a)
	O_2^-	control	O_2^-	control	(b)
0	114±9	116±6	103±7	103±4	
7	129±12	115±12	82±6	91±8	
14	88±12	104±15	52±9	81±7	
21	98±10	100±6	48±5	96±8	
28	86±13	108±11	41±5	89±9	

(a) mean and std. dev . of counts per field

(b) treatment of cells

Discussion.

This chapter adds to the body of evidence supporting a role for reactive oxygen intermediates in lymphocyte transformation and it demonstrates, by direct activation of lymphocytes rather than blocking of mitogenesis, this involvement. Although the use of potassium superoxide and superoxide dismutase suggest that the O_2^- anion itself may be responsible for the phenomenon, it is equally likely that an O_2^- -derived species is the causative agent. A current lack of suitable scavengers of these species has prevented the resolution of this question.

One entertaining possibility which has come out of the work on ROI is that they could be fundamentally involved in the process of lymphocyte transformation, rather than acting as mitogens in the manner of plant lectins. This idea is supported by the findings of Wrogemann et al (1978 and 1980) and Novogrodsky et al (1982) who have used scavengers of ROI to inhibit mitogenic transformation. As yet, no studies have been done on the effects of ROI scavengers on antigenic stimulation, which is much more complex than mitogenic stimulation but work in this area will be of importance in the future.

If this theory is correct, it may explain an interesting observation by Baeteman et al (1983). They have shown that trisomy-21 patients have abnormally high levels of Cu-SOD and often suffer from clinical immunodeficiencies. It is tempting to speculate that these deficiencies result from an over-effective scavenging of superoxide. Curtis et al (1973) showed that

oxidation of a surface to increase the number of hydroxyl groups enhanced the adhesion of cells to that surface. It is possible that a similar type of reaction between the perhydroxyl radical ($HO_2^{\cdot}$) (a known oxidizing agent) and components of the plasma membrane cause the observed superoxide-mediated increase in cell adhesion. This may tie in with the mechanism of ROI induced lymphocyte activation: the oxidation of sulphydryl groups of membrane proteins by free radicals may cause cross linkage of the relevant surface receptors, bringing about activation. A model of this is the cross linkage of spectrin in erythrocytes (Chiu et al, 1982). An alternative explanation is that lipid peroxidation, which occurs readily in unsaturated fatty acids and is propogated by a free radical cascade, brings about changes in membrane structure and function, causing cell activation.

The work presented in this thesis concerns the adhesive interactions of the cells of the immune system. Evidence is presented for a possible role for the major histocompatibility complex antigens regulating cell adhesiveness. Although the effects are small, it is possible that they represent the fine control of adhesion of these cells. The system used to study these effects, syngeneic and allogeneic combinations of cells and histocompatibility antigens, is totally artificial but may parallel interactions either between immune cells and targets with virally or otherwise modified MHC antigens, or between lymphocytes and antigen presenting cells in the generation of the immune response. In the latter case, class II (Ia) antigens are most relevant. It has been shown here that soluble Ia antigens can be attached to allogeneic cells in such a way that they are antigenically recognizable by antibodies and can alter mixed lymphocyte responses.

Lymphocyte cell surfaces are also affected by treatment with solutions of potassium which increases their adhesiveness and causes their adhesion. This activation can be demonstrated by addition of exogenous superoxide or stimulation of endogenous production and, since the reactive oxygen intermediates responsible are found in vivo, it may be a physiologically important phenomenon.

It is possible that histocompatibility antigen and/or foreign antigen bridges serve to bring cooperating cells into close contact and to generate a proliferative signal. Reactive oxygen intermediates, both by increasing the the adhesiveness of these

cells and by directly activating them may represent a complimentary lymphocyte activation system.

Thus, it is possible that the adhesion of the cells of the immune system may help in its regulation.

REFERENCES.

Aarden,E., Burnner,T.K., Cerrotini,J.C., Dayer,J.M., de Weck,A.L., Dinarello,C.A., diSabato,G., Farrar,J.J., Gery,I., Gillis,S., Handschumaker,R.E., Henney,C.S., Hoffmann,M.K., Koopman,W.J., Karane, S.M.,Lachman,L.B., Lefkovitz,I., Mishel,R.I., Mizel,S.B., Oppenheim,J.J., Paetkau,V., Plate,J., Rollingoff,M., Rosenstreich,D., Rosenthal,A.S., Rossenwasser,L. J., Schimpl,A., Shin,H.S., Simon,P.L., Smith,K.A., Wagner,H., Watson,J.D., Weiber,E. and Wood,D.D. (1977). Letter to the editor. Revised nomenclature for antigen-non-specific T cell proliferation and helper factor.J.Immunol.123,2928-2929.

Abercrombie,M. and Heaysman,J.E.M. (1954). Observations on the social behaviour of cells in tissue culture II."Monolayering of fibroblasts." Exp.Cell Res.,6,293-306.

Acuto,E., Hussey,R.E., Fitzgerald,K.A., Protentis,J.P., Meller,S.C., Schlossman,S.F. and Reinherz,E.L. (1983). The human T cell receptor: appearance in ontogeny and biochemical relationship of and subunits on IL-2 dependant clones and T cell tumours. Cell,34,717-726.

Agro,A.F., Conti, P., Cifone,M.G., Argeletti,P.U. and Rotilio,G. (1980). Effect of hydrogen peroxide on lymphocytes. In Bannister, W.H. and Bannister,J.V.(Eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland.

Aplin,J.D. and Hughes,R.C. (1981). Modification of fibroblast surface amines alters receptor-mediated cell spreading on protein coated substrates but not absorptive endocytosis. J.Cell Sci.,49,283-297.

Baboir,B.M. (1978). Oxygen-dependant microbial killing by phagocytes. New Eng.J.Med.,298,659-668.

Bach,F,H., Widmer,M.B., Bach,M.L. and Klein,J. (1972). Serologically defined and lymphocyte-defined components of the major histocompatibility complex in mice. J.Exp.Med.,136,1430-1444.

Bachur,N.R., Gordon,S.L. and Gee,M.V. (1978). A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res., 38,1745-1750.

Baeteman,M.A., Baret,A., Courtiere,J.-P., Rebuffel,P. and Mattei,J.F. (1983). Immunoreactive Cu-SOD and Mn-SOD in lymphocyte subpopulations from normal and trisomy-21 subjects according to age. Life Sciences,32,895-902.

Baldridge,C.W. and Gerard,R.W. (1933). The extra respiration of phagocytosis. Am.J.Physiol.,103,235-236.

Bartlett,P.F. and Edidin,M. (1978). Effect of H-2 gene complex on rates of fibroblast intercellular adhesion. J.Cell Biol.,77,377-388.

Baxevanis,C.N., Nagy,Z.A. and Klein,J. (1981). A novel type of T-T cell interaction removes the requirement for I-B region in the H-2 complex. Proc.Natl.Acad.Sci.USA.78,3809-3813.

Bell,G.I. (1978). In Bell,G.I., Perelson,A.S. and Pimbley,G.H. Jr.(Eds.), Theoretical Immunology, Marcel Decker,New York.

Bell,G.I. (1978). Models for specific adhesion of cell to cells. Science,200,618-629.

Bennet,D. (1980). The T complex in the mouse: an assessment after 50 years of study. In The Harvey Lectures. Series 74.(New York Academic Press).pp1-21.

Berton,G. and Gordon,S. (1983). Superoxide release by peritoneal and bone-marrow derived mouse macrophages. Modulation by adherence and cell activation. Immunol.,49,693-704.

Bodmer,W.F.(1972). Evolutionary significance of the HL-A system. Nature,237,139-145.

Bors,W., Saran,M. and Czapski,G. (1980). The nature of intermediates during biological oxygen activation. In Bannister,W.H. and Bannister,J.V.(Eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland, New York.

Calderon,J. and Unanue,E.R. (1974). The release of antigen molecules from macrophages: charecterization of the phenomena. J.Immunol.,112,1804-1814.

Carson,S., Vogin,E.E., Huber,W. and Schulte,T.L. (1973). Safety tests of orgotein, an anti-inflammatory protein. Toxicol.Appl.Pharmacol.,26,184-202.

Charron,D.J. and McDevitt,H.O. (1980). Characterization of HLA-D region antigens by 2-dimensional gel electrophoresis. J.Exp.Med.,152,18s-36s.

Chesnut,R.W., Colon,S.M. and Grey,H.M. (1982). Antigen presentation by normal B cells, B cell tumours and macrophages: functional and biochemical comparison. J.Immunol.,128,1764-1768.

Chien,Y.-L., Gascoigne,N.R.J., Kavalar,J., Lee,N.E. and Davis,M.M. (1984). Somatic recombination in a murine T-cell receptor gene. Nature,309,322-326.

Chiller,J.M., Habicht,G.S. and Weigle,W.O. (1970). Cellular sites of immunological unresponsiveness. Proc.Natl.Acad.Sci.USA,65,551-556.

Chiu,D., Lubin,B. and Shohet,S.B. (1976). Peroxidative reactions in red cell biology. In Pryor,W.A.(ed.), Free Radicals in Biology Volume V. Academic Press, New York.

Cook,R.G., Uhr,J.W., Capra,J.D. and Vitetta,E.S. (1978). Structural studies on murine Ia alloantigens II. Molecular weight charecterization of the products of the I-A and I-E/C subregions. J.Immunol.,121,2205-2212.

Cook,R.G., Vitetta,E.S., Uhr,J.W. and Capra,J.D. (1979). Structural studies on the murine Ia alloantigens-III. Tryptic peptide comparisons of allelic products of the I-E/C subregions. Mol.Immunol.,16,29-35.

Cowing,C., Schwartz,B.D. and Dickler,H.B. (1978). Macrophage Ia antigens I. Macrophage populations differ in their expression of Ia antigens. J.Immunol.,120,378-384.

Cullen,S.E., Freed,J.H. and Nathenson,S.G. (1976). Structural and serological properties of murine Ia alloantigens. Transplant.Rev.,30,236-270.

Cullen,S.E., Kindle,C.S. and Littman,D.R. (1979). Structural comparison of murine Ia antigens determined by the I-A and I-E subregions. J.Immunol.,122,855-859.

Cullen,S.E., Shreffler,D.C., Kindle,C.S. and Davis,C.S. (1980). Identity of molecules bearing murine alloantigenic specificities Ia.7 and Ia.22: evidence for a single type of I-E molecule in homozygotes. Immunogenet.,11,535-547.

Cunningham,B.A., Wang J.L., Berggard,I. and Peterson,P.A. (1973). The complete amino acid sequence of β_2-microglobulin. Biochemistry,12,4811-4821.

Curman B., Ostberg,L. and Peterson,P.A. (1978). Incorporation of murine MHC antigens into liposomes and their effect in the secondary mixed lymphocyte reaction. Nature, 22,545-547.

Curtis,A.S.G. (1969). The measurement of cell adhesiveness by an absolute method. J.Embryol.Exp.Morph.,22,305-325.

Curtis,A.S.G. (1978). Cell-cell recognition : positioning and patterning systems. In Curtis,A.S.G.(ed.) Cell-Cell Recognition. (32nd Symposium of the Society for Experimental Biology). Cambridge University Press, Cambridge.

Curtis,A.S.G. and Davies,M.D.J. (1981). H-2D antigens released by thymocytes and cell adhesion. J.Immunogenet.,8,367-377.

Curtis,A.S.G. (1982). The genetic basis of cell behavior in lymphocytes and other cells. In Bellairs,R., Curtis,A.S.G. and Dunn,G.A.(eds.), Cell Behaviorn, Cambridge University Press, Cambridge.

Curtis,A.S.G. and de Souza,M. (1975). Lymphocyte interactions and positioning. Cell Immunol.,19,282-297.

Curtis,A.S.G. and Renshaw,R.M. (1982). Lymphocyte-endothelial interactions and histocompatibility restriction. In Nieuwenhuis, van den Broek and Hanna, In vivo immunology, Plenum Publishing Group.

Curtis,A.S.G., Davies,M. and Wilkinson,P.C. (1979). New evidence of control mechanisms in lymphocyte traffic. In Muller-Ruchholtz,W. and Muller-Hermalink,H.K.(eds.), Function and Structure of the Immune System. Plenum Press, London, New York.

Curtis,A.S.G., Forrester,J.V., McInnes,C. and Lawrie, F. (1983). Adhesion of cells to polystyrene surfaces. J.Cell Biol.,97,1500-1506.

Dahinden,C.A., Fehr,J. and Hugli,T.E. (1983). Role of cell surface contact in the kinetics of superoxide production by granulocytes. J.Clin.Invest.,72,113-121.

Day,C.E. and Jones,P.P. (1983). The gene encoding the Ia antigen-associated invarient chain (Ii) is not linked to the H-2 complex. Nature,302,157-159.

Delovitch,T.L., Harris,J.F., Batistella,R. and Kaufman,K. (1982). Role of Ia antigens in graft versus host reactions II. Molecules and functional analysis of T cell alloreactivity by the charecterization of host Ia antigens on alloactivated donor T cells. J.Exp.Med.,155,61-82.

Demant,P., Capkova,J., Hinzovna,E. and Vorakova,B. (1973). The role of histocompatibility-2-linked Ss-Slp region in the control of mouse complement. Proc.Natl.Acad.Sci.USA,70,863-866.

Doherty,P.C. and Zinkernagel,R.M. (1975). H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. J.Exp.Med.,141,502-507.

Dresser,D.W. and Mitchison,N.A. (1968). The mechanism of immunological paralysis. Adv.Immunol.,8,129-181.

Esnouf,P.M., Gainey,A.I., Green,M.R., Hill,H.A.O., Okolow-Zubkowska,M.J. and Walter,S.J. (1980). The production and utilization of superoxide during the carboxylation of glutamyl residues in preprothrombin. In Bannister,W.H. and Bannister,J.V.(eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland, New York.

Farr,A.G., Kiely,J.-M. and Unanue,E.R. (1979a). Macrophage-T cell interactions involving Listeria monocytogenes- role of the H-2 gene complex. J.Immunol.,122,2395-2400.

Farr,A.G., Wechter,W.J., Kiely.J.-M. and Unanue,E.R. (1979b). Induction of cytocidal macrophages after in vitro interactions between Listeria-immune T cells and macrophages- role of the H-2 gene complex. J.Immunol.,122,2405-2412.

Farrar,J.J., Benjamin,W.R., Hilfeker,M.L., Howard,M., Farrar,W.L. and Fuller-Farrar,J. (1982). The biochemistry, biology and role of interleukin 2 in the induction of cytotoxic T cells and antibody-forming cell responses. Immunological Rev.,63,129-166.

Feldmann,M. and Basten,A. (1972). Specific colaboration between T and B lymphocytes across a cell impermeable membrane. Nature New Biol.,237,13-15.

Ferrone,S., Allison,J.P. and Pellegrino,M.A. (1978). Human DR (Ia-like) antigens: biochemical and molecular profile. Cont.Top.Mol.Immunol.,7,239-281.

Fraker,P.J. and Speck,J.C. (1978). Protein and cell membrane iodinations with a sparingly soluble chloramine 1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril. Biochem.Biophys.Res.Comm., 80,849-857.

Freed,J.H. and Nathenson,S.G. (1978). Similarities of carbohydrate structures of H-2 and Ia glycoproteins. J.Immunol.,119,477-482.

Frelinger,J.A., Niederhuber,J.E. and Schreffler,D.C. (1975). Inhibition of immune responses in vitro by specific antiserums to Ia antigens. Science,188,268-270.

Fridovitch,I. (1970). Quantitative aspects of the production of superoxide anion by milk xanthine oxidase. J.Biol.Chem.,245,4053-4057.

Fridovitch,I. (1976). Oxygen radicals, hydrogen peroxide and oxygen toxicity. In Pryor,W.A. (ed.), Free Radicals in Biology, Volume I.

Galeotti,T., Bartoli,G.M., Bartoli,S. and Bertoli,E. (1980). Superoxide radicals and lipid peroxidation in tumour microsomal membranes. In Bannister,W.H. and Bannister,J.V.(eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland.

Geib,R., Goldberg,E.H. and Klein,J. (1977). Membrane-bound H-2 and H-Y antigens move independantly of each other. Nature,270, 352-354.

Goetze,D. (ed) (1977). The Major Histocompatibility System in Man and Animals. Springer Verlag, Berlin.

Green,T.R., Wirtz,M.K. and Wu,D.E. (1983a). Delineation of the catalytic components of the NADPH-dependant O_2^- generating oxidoreductase of human neutrophils. Biochim.Biophys.Res.Comm., 110,873-879.

Green,T.R., Wirtz,M.K. and Wu,D.E. (1983b). The O_2^- generating oxidoreductase of human neutrophils : evidence of an obligatory requirement for calcium and magnesium for expression of catalytic activity. Biochim.Biophys.Res.Comm.,110,973-978.

Greineder,D.K., Shevach,E.M. and Rosenthal,A.S. (1976). Macrophage-lymphocyte interactions. III. Site of alloantiserum inhibition of T-lymphocyte proliferation induced by allogeneic or aldehyde-bearing cells. J.Immunol.,117,1261-1266.

Habu,H. (1979). Clinicohistopathological study on "borderline atypical epithelial lesions" of the human stomach with special reference to histogenesis. (Eng.Abstr.). Nippon Shokakibyo Gakka Zasshi,76,23-37.

Halliwell,B., Richmond,R., Wong,S.F. and Gutteridge,J.M.C. (1980). The biological significance of the Haber-Weiss reaction. In Bannister,W.H. and Bannister,J.V.(eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland.

Hansen,T.H., Ozato,K., Melino,M.R., Coligan,J.E., Kindt,T.J., Jandinski,J.J. and Sachs,D.H. (1981). Immunochemical evidence in two haplotypes for at least three D region encoded molecules D, L and R. J.Immunol.126,1713-1716.

Haston,W.S. (1979). A study of lymphocyte behaviour in cultures of fibroblast-like lymphoreticular cells. Cell Immunol.,45,74-84.

Hiramatsu,K., Ocki,A., Miyatani,S., Segawa,A. and Toda,T. (1982). Monoclonal antibodies against I-region gene products expressed only on mature functonal T cells. Nature,296,666-668.

Hedrick,S.M., Cohen,D.I., Nielsen,E.A. and Davis,M.M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature,308,149-153.

Heber-Katz,E. and Wilson,D.B. (1975). Collaboration of allogeneic T and B lymphocytes in the primary antibody response to sheep erythrocytes in vitro. J.Exp.Med.,142,928-935.

Heyningen,V.van, Guy,K. and Steel,C.M. (1982). Human MHC class II molecules as differentiation markers. Immunogenetics,16,459-469.

Howard,M., Farrar,J., Hilfiker,M., Johnson,B., Takatsu,K., Hamaoka,T. and Paul,W. (1982). Identification of a T cell-derived B cell growth factor distinct from interleukin 2. J.Exp.Med.155, 914-924.

Howie,S. and Feldmann,M. (1977). In vitro studies on H-2-linked unresponsiveness to synthetic polypeptides III, Production of antigen-specific T helper cell factor to (T,G)-A--L. Eur.J.Immunol.,7,417-421.

Hudson,L. and Hay,F.C. (1980). Practical Immunology, Blackwell Scientific Publications, Oxford.

Ivanyi,D. and Demant,P. (1979). Complex genetic effect of B10.D2(M504) (H-2dm1) mutation. Immunogenetics,8,539-550.

Ivanyi,D., Gregorova,S. and Mickova,M. (1972a). Genetic differences in thymus, lymph nodes, testes and vesicular gland weights among inbred mouse strains. Association with H-2 system. Folia Biol.(Praha),18,81-97.

Jenkinson,E.S., van Ewijk,W. and Owen,J.J.T. (1981). Major histocompatibilty complex expression on the epithelium of the developing thymus in normal and nude mice. J.Exp.Med.,153,280-292.

Ivanyi,D., Hemysl,R., Starka,L. and Mickova,M. (1972b). Genetic association between H-2 gene and testosterone metabolism in mice. Nature New Biol.,238,280-281.

Jones,P.P., Murphy,D.B., Hewgill,H. and McDevitt,H.O. (1978). Detection of a common polypeptide chain in I-A and I-E subregion immunoprecipitates. Mol.Immunol.16,51-60.

Julius,M.H., Chiller,J.M. and Sidman,C.L. (1982). Major histocompatibility complex-restricted cellular interactions determining B cell activation. Eur.J.Immunol.,12,627-633.

Kappler,J.W., Skidmore,B., White,J. and Marrack,P. (1981). Antigen-inducible, H-2 restricted, interleukin 2-producing T cell hybridomas. Lack of independant antigen and H-2 recognition. J.Exp.Med.,153,1198-1214.

Kappler,J. Kubo,R., Haskins,K., White,J. and Marrack,P. (1983). The mouse T cell receptor : comparison of MHC-restricted receptors on two T cell hybridomas. Cell,34,727-737.

Katz,D.H., Hamaoka,T. and Benacerraf,B. (1973). Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. J.Exp.Med.,137,1405-1418.

Keele,B.B.Jr., McCord,J.M. and Fridovitch.I. (1970). Superoxide dismutase from Escherichia coli B. J.Biol.Chem.,245,6176-6186.

Kellogg,E.W.III and Fridovitch,I. (1975). Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J.Biol.Chem.,250,8812-8817.

Klebanoff,S.J. and Clark,R.A. (1978). In The Neutrophil: Function and Clinical Disorders. pp283-488. Elsevier/North Holland, New York.

Klein J. (1975). Biology of the Mouse Histocompatibility Complex. (Berlin: Springer Verlag).

Klein,J. (1979). The major histocompatibility complex of the mouse. Science,203,516-521.

Klein,J. (1982). Evolution and function of the MHC. In Parham,P. and Strominger,J.L. (eds.). Histocompatibility antigens, structure and function. Receptors and Recognition. Series B, Volume 14, Chapman and Hall, London.

Koch,G.L.E. and Smith M.J. (1978). An association between actin and the major histocompatibility complex H-2. Nature,273,274-278.

Koch,N., Arnold,B., Hammerling,G.J., Heuer,J. and Kolsch,E. (1983). Structure comparison of Ia antigens produced by a cloned murine T suppressor cell line with B cell-derived I-A. Immunogenetics,17,497-505.

Koch,N., Koch,S. and Hammerling,G. (1982). Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. Nature,299,644-645.

Koszinowiski,U. and Ertl,H. (1975). Altered serological and cellular reactivity to H-2 antigens after target cell infection with vaccinia virus. Nature,257,596-597.

Krinsky,N.I. (1974). Singlet excited oxygen as a mediator of the antibacterial action of leucocytes. Science,180,363-365.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature,227,680-684.

Langman,R.E. and Cohn,M. (1981). Why the MHC is important to the immune system. Transplant.Proc.,13,1797-1799.

Lilley,F. (1971). The influence of H-2 type on Gross virus leukaemogenesis in mice. Transplant.Proc.,3,1239-1242.

Lorber,M.I., Loken,M.R., Stall,A.M. and Fitch,F.W. (1982). I-A antigens on cloned alloreactive murine T lymphocytes are acquired passively. J.Immunol.,128,2798-2803.

Matzinger,P. (1981). A one-receptor view of T-cell behaviour. Nature,292,497-501.

Mann,D.L. and Abelson,L. (1980). Monocyte function in mixed lymphocyte reactions. Cell Immunol.,56,357-364.

McCord,J.M. (1974). Free radicals and inflammation I. Protection of synovial fluid by superoxide dismutase. Science,185,529-531.

McCord,J.M. and Fridovitch,I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J.Biol.Chem., 244,6049-6055.

McCord,J.M., English,D.K. and Petrone,W.F. (1980). A role for superoxide in granulocyte-mediated inflammation. In Bannister, W.H. and Bannister,J.V.(eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland.

McDevitt,H.O. and Chinitz,A. (1969). Genetic control of the antibody response: relationship between immune response and histocompatibility (H-2) type. Science,163,1207-1208.

McNicholas,J.M., King,D.P. and Jones,P.P. (1983). Biosynthesis and expression of Ia and H-2 antigens on a macrophage cell line are stimulated by products of activated spleen cells. J.Immunol.,130,449-456.

Meo,T., David,C.S. and Shreffler,D.C. (1976). In Katz,D.H. and Benacerraf,B. (eds).The Role of Products of the Histocompatibility Gene Complex in Immune Rsponses. Academic Press, New York.

Meruelo,D. and Edidin,M. (1975). Association of mouse liver 3'5'cyclic monophosphate (cyclic AMP) with histocompatibility-2 genotype. Proc.Natl.Acad.Sci.USA,72,2644-2648.

Miyatani,S., Hiramatsu,K., Nakajima,P.B., Owen,F.L. and Tada,T. (1983). Structural analysis of antigen-specific Ia-bearing regulatory T cell factors : gel electrophoretic analysis of the antgen-specific augmenting T cell factor. Proc.Natl.Acad.Sci.USA, 6336-6340.

Nagy,Z.A., Elliott,B.E., Nabholz,M., Krammmer,P.H. and Pernis,B. (1976). Specific binding of alloantigens to T cells activated in the mixed lymphocyte reaction. J.Exp.Med.,143,648-659.

Nagy,Z.A., Elliott,B.E., Carlow,D.A. and Rubin.B. (1982). T cell idiotypes recognizing self minor histocompatibility complex molecules: H-2 specificity, allotype linkage and expression on functional T cell populations. Eur.J.Immunol.,12,393-400.

Nairn,R., Yamaga,K. and Nathenson,S.G. (1980). Biochemistry of gene products from murine MHC mutants. Ann.Rev.Genet.,14,241-277.

Nakagawa,A., deSantis,N.M., Noguiera,N. and Nathan,C.F. (1982). Lymphokines enhance the capacity of human monocytes to secrete reactive oxygen intermediates. J.Clin.Invest.,70,1042-1048.

Nathenson,S.G. and Cullen,S.G. (1974). Biochemical properties and immunochemical-genetic relationships of mouse H-2 alloantigens. Biochim.Biophys.Acta.344,1-25.

Nathenson,S.G., Uehara,H. and Ewenstein,B.M. (1981). Primary srtuctural analysis of the transplantation antigens of the murine H-2 major histocompatibility colmplex. Ann.Rev.Biochem.,50,1025-1052.

Nixon,D.F., Pan-Yun-Ting,J. and Frelinger,J.A. (1982). Ia antigens on non-lymphoid tissues. Their origins and functions. Immunol.Today,3,339-342.

Novogrodsky,A. and Katchalski,E. (1972). Membrane site modified on induction of the transformation of lymphocytes by periodate. Proc.Natl.Acad.Sci.USA,70,1824-1827.

Novogrodsky,A. and Katchalski,E. (1973). Induction of lymphocyte transformation by sequential treatment of neuraminidase and galactose oxidase. Proc.Natl.Acad.Sci.USA,70,1824-1827.

Novogrodsky,A., Ravid,A., Rubin,A.L. and Stenzl,K.H. (1982). Hydroxyl radical scavegers inhibit lymphocyte mitogenesis. Proc.Natl.Acad.Sci.USA,79,1171-1174.

Oakley,B.R., Kirsch,D.R. and Morris,N.R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal.Biochem.,105,361-363.

O'Brien,P.J. and Rahimtula,A. (1976). The possible involvement of a peroxidase in prostoglandin biosynthesis. Biochem.Biophys.Res. Comm.,70,832-845.

Ohno,S. (1971). The original function of MHC antigens as the general plasma membrane anchorage site for organogenesis-directing proteins. Immunol.Rev.,33,59-69.

Orr,H.T. (1982). The major histocompatibility complex: analysis at the protein and DNA levels. In Parham,P. and Strominger,J.(Eds). Histocompatibility Antigens. Structure and Function. Receptors and Recognition SeriesB.Volume14,pp1-51.

Orr,H.T., Lopez de Castro,J.A., Lancet,D. and Strominger,J.L. (1979). Complete amino acid sequence of a papain-solubilized histocompatibility antigen, HLA-B7. 2. Sequence determination and search for homologies. Biochemistry,18,5711-5716.

Oss,van C.J., Gillman,C.F. and Neumann,A.W. (1975). Phagocytic Engulfment and Cell Adhesiveness as Cellular Surface Phenomena. Miorganisms and Infectious Diseases Volume 2, Marcel Decker Inc.,New York.

Ouderra,F.J.van der, Buytenhek,M., Nugteren,D.H. and Dorp,D.A.van (1977). Purification and charecterization of prostoglandin endoperoxidase synthetase from sheep vesicular glands. Biochim. Biophys.Acta.,487,315-331.

Parham,P., Albert,B.N., Orr,H.T. and Strominger,J.L. (1977). Carbohydrate moiety of HLA antigens. J.Biol.Chem.,252,7555-7567.

Paul,W.E., Shevach,E.M., Pickerall,S., Thomas,D.W. and Rosenthal, A.S. (1977). Independant populations of primed F1 guinea-pig T lymphoytes respond to antigen-pulsed parental peritoneal exudate cells.J.Exp.Med.,145,618-630.

Pecknicova,J., Matousek,V. and Viklily,V. (1981). On the mechanism of intercellular adhesion. Folia Biol.(Praha),27,284-288.

Pickel,K., Hammerling,U. and Hoffmann,M.K. (1976). Ly phenotype of T cell replacing factor. Nature,264,72.

Ploegh,H.L., Orr,H.T. and Strominger,J.L. (1981a). Major histocompatibility antigens. The human (HLA-A,-B,-C) and murine (H-2K,D) class I molecules. Cell,24,287-299.

Ploegh,H.L., Orr,H.T. and Strominger,J.L. (1981b). Biosynthesis and cell surface localization of nonglycosylated human histocompatibility antigens. J.Immunol.,126,270-275.

Pober,J.S. and Gimborne,M.A. (1982). Expression of Ia-like antigens by human vascular endothelial cells is inducible in vitro: demonstration by monoclonal antibody binding and immunoprecipitation. Proc.Natl.Acad.Sci.USA,79,6641-6645.

Rask,L., Limblom,J.B. and Peterson,P.A. (1974). Subunit structure of H-2 alloantigens. Nature,249,833-834.

Richman,L.K., Klingenstein,R.J., Richman,J.A., Strober,W. and Berzofsky,J.A. (1979). The murine Kupffer cell I. Charecterization of the cell serving accessory function in antigen-specific T cell proliferation. J.Immunol.,123,2602-2609.

Robinson,P.J., Graf,L. and Sege,K. (1981). Two allelic forms of mouse β_2-microglobulin. Proc.Natl.Acad.Sci.USA,774239-4243.

Root,R.K. and Metcalf,J.A. (1977). H_2O_2 release from human granulocytes during phagocytosis : relationship to superoxide anion formation and cellular catabolism of H_2O_2 : studies with normal and cytochalasin-B treated cells. J.Clin.Invest.,60,1266-1279.

Rosenthal,A.S. (1980). Regulation of the immune response - role of the macrophage. New Eng.J.Med.,303,1153-1156.

Rosenthal,A.S. and Shevach,E.M. (1973). Function of macrophages in antigen recognition by guinea-pig T lymphocytes. I.Requirement of histocompatible macrophages and lymphocytes. J.Exp.Med.,138, 1194-1211.

Rothbard,J.B., Hopp,T.P., Edelman,G.M. and Cunningham,B.A.(1980). Structure of the heavy chain of the H-2Kk histocompatibility antigen. Proc.Natl.Acad.Sci.USA,77,4239-4243.

Sandrin,M.S., Vaughan,H.A., McKenzie,I.F.C., Higgins,T.J. and Parish,C.R. (1981). The second class of Ia antigens: the Ia^+ glycolipids. Transplant.Proc.,13,1014-1016.

Schwartz,B.D. and Nathenson,S.G. (1971). Isolation of H-2 alloantigens solubilized by detergent NP-40. J.Immunol.,107,1363-1367.

Schwartz,B.D., Kask,A.M., Paul,W.E., Geczy,A.F. and Shevach,E.M. (1977). The guinea-pig I region. I. A structural and genetic analysis. J.Exp.Med.,146,547-560.

Schwartz,D.H., David,C.S., Dorf,M.E., Benacerraf,B. and Paul,W.E. (1978). Inhibition of dual Ir gene-controlled T-lymphocyte proliferative responses to poly(Glu56Lys35Phe9) with anti-Ia antisera directed against products of either I-A or I-C subregion. Proc.Natl.Acad.Sci.USA,75,2387-2391.

Schwartz,R.H. (1978). A clonal deletion model for Ir gene control of the immune response. Scand.J.Immunol.,7,3-10.

Schwartz,R.H., Fathman,G. and Sachs,D.H. (1976). Inhibition of stimulation in murine mixed lymphocyte cultures with an alloantiserum directed against a shared Ia determinant. J.Immunol.,116,929-935.

Shearer,G.M., Rehn,T.G. and Garbarino,C.A. (1975). Cell-mediated lympholysis of triphenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. J.Exp.Med.141,1348-1364.

Shevach,E.M., Finkelman,F. and Ben-Sasson,S.Z. (1975). Ir genes and antigen recognition. Ann.N.Y.Acad.Sci.,249,541-547.

Shimada,A. and Nathenson,S.G. (1969). Murine histocompatibility -2 (H-2) alloantigens. Purification and some general properties of soluble products from H-2b and H-2d genotypes released by papain digestion of membrane fractons. Biochemistry,8,4048-4062.

Shortman,K. and Goldstein,P. (1979). Target cell recognition by cytolytic T cells: different requirements for the formation of strong conjugates or for proceeding to lysis. J.Immunol.,123,833-839.

Silver,J. and Hood,L. (1976). Detergent-solubilized H-2 alloantigen is associated with a small molecular weight polypeptide. Nature,249,764-7654.

Simon,P.L., Farrar,J.J., and Kind,P.D. (1977). Biochemical relationship between murine immune interferon and a killer cell helper factor. J.Immunol.,122,127-132.

Smith,K.A., Gilbride,K.J. and Favate,M.F. (1980). Lymphocyte activating factor promotes T cell growth factor production by cloned murine lymphoma cells. Nature,287,853-857.

Springer,T.A. and Strominger,J.L. (1976). Detergent-solubilized HLA antigens contain a hydrophilic region at the COOH-terminus and a penultimate hydrophobic region. Proc.Natl.Acad.Sci.USA,73, 2481-2486.

Sredni,B. and Schwartz,R.H. (1981). Antigen-specific, proliferating clones. Methodology, specificity MHC restriction and alloreactivity. Immunological Rev.,54,187-223.

Steeg,P.S., Moore,R.N. and Oppenheim,J.J. (1980). Regulation of murine macrophage Ia-antigen expression by products of activated spleen cells. J.Exp.Med.,152,1734-1744.

Steinmetz,M., Moore,K.W., Frelinger,J.G., Sher,B.T., Shen,F.-W., Boyse,E.A. and Hood,L. (1981). A pseudogene homologous to mouse transplantaton antigens : transplantation antigens are encoded by eight exons that correlate with protein domains. Cell,25,683-692.

Steinmetz,M., Minard,K., Horvath,S., McNicholas,J., Srelinger,J., Wake,C., Long,E., Mach,B. and Hood,L. (1982a). A molecular map of the immune response region from the major histocompatibility complex of the mouse. Nature ,300,35-42.

Steinmetz,M., Winoto,A., Minard,K. and Hood,L. (1982b). Cluster of genes encoding mouse transplantation antigens. Cell,28,489-498.

Sunshine,G.H., Gold,D.P., Wortis,H.H., Marrack,P. and Kappler, J.W. (1983). Mouse spleen dendritic cells present soluble antigen to antigen-specific T cell hybridomas. J.Exp.Med.,158,1745-1750.

Suzuki,H. and Kakinuma,K. (1983). Evidence that NADPH is the actual substrate of the oxidase responsible for the "respiratory burst" of phagocytosing polymorphonuclear leucocytes. J.Biochem., 93,709-715.

Tada,T. and Okumura,K. (1979). The role of antigen-specific T cell factors in the immune response. Adv.Immunol.,28,1-87.

Tada,T., Taniguchi,M. and David,C.S. (1976). Properties of the antigen-specific suppressive T cell factor in the regulation of antibody response in the mouse IV. Special subregion assignment of the gene(s) that code for the suppressive T cell factor in the H-2 histocompatibility complex. J.Exp.Med.,144,713-725.

Thomas,D.W. and Shevach,E.M. (1977). Nature of antigenic complex recognized by T lymphocytes II. T cell activation by direct modification of macrophage histocompatibility antigens. J.Exp.Med.,145,907-915.

Towbin,H., Staehelin,T. and Gordon,J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Natl.Acad.Sci.USA, 76,4350-4354.

Treissman,M. (1981). The significance of immunity restriction by the major histocompatibility complex and of the occurence of high polymorphism at MHC loci : two hypothesis. J.Theor.Biol.,89,409-421.

Twomey.J.J., Sharkey,O.Jr., Brown,J.A., Laughter,A.H. and Jordan, P.H.Jr. (1970). Cellular requirement for mitotic response in allogeneic mixed leucocyte cultures. J.Immunol.,104,845-853.

Ursini,F., Bindoli,A., Cavallini,L. and Maiorino,M. (1980). Hydrogen peroxide and singlet oxygen in lipid peroxidation. In Bannister,W.H. and Bannister,J.V.(eds.), Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Elsevier/North Holland.

Virtanen,I., Vartio,T., Badley,R.A. and Lehto,V.-P. (1982). Fibronectin in adhesion, spreading and cytokeletal organization of cultured fibroblasts. Nature,298,660-663.

Visser,J. (1976). The adhesion of colloidal polystyrene particles to cellophane as a function of pH and ionic strength. J.Coll.Interface Sci.,55,664-677.

Walsh,F.S. and Crumpton,M.J. (1977). Orientation of cell surface antigens in the lipid bilayer of lymphocyte plasma membrane. Nature,308,307-311.

Watson,J. (1977). Continuous proliferation of immune antigen-specific helper T lymphocytes in culture. J.Exp.Med.,150,1510-1519.

Williams,A.F. (1984). The T lymphocyte antigen receptor- elusive no more. Nature,308,108-109.

Winchester,R.J. and Kunkel,H.G. (1979). The human Ia system. Adv.Immunol.,28,221-191.

Winchester,R.J., Wang,C.-Y., Gibofsky,A., Kunkel,H.G., Lloyd,K.O. and Old,L.J. (1978). Expression of Ia-like antigens on activated human malignant melanoma cell lines. Proc.Natl.Acad.Sci.USA,75, 6235-6239.

Wroggman,K., Weidemann,M.J., Peskar,B.A., Staudinger,H., Rietschel,E.T. and Fischer,H. (1978). Chemiluminescence and immune activation I. Early activation of rat thymocytes can be monitered in chemiluminescence measurements. Eur.J.Immunol.,8, 749-752.

Wroggmann,K., Weidemann,M.J., Ketelsen,U.-P., Weherle,H. and Fischer,H. (1980). Chemiluminesence and immune cell activation II. Enhancement of concanavlin-A-induced chemiluminescence following in vitro preincubation of rat thymocytes ; dependancy on macrophage-lymphocyte interaction. Eur.J.Immunol.,10,36-40.

Yanagi,Y., Yoshikai,Y., Leggett,K., Clark,S.P., Aleksander,I. and Mak,T.W. (1984). A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature,308,145-149.

Yost,F.J.Jr., and Fridovitch,I. (1973). An iron-containing superoxide dismutase from Escherichia coli. J.Biol.Chem.,248, 4905-4908.

Zeleny,V., Matousek,V. and Lengerova,A. (1978). Intercellular adhesiveness of H-2 identical and disparate clones. J.Immunogenet.,5,41-47.

Ziegler,K. and Unanue,E.R. (1981). Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. J.Immunol.127,1869-1875.

Zinkernagel,R.M. (1978). Thymus factors and reconstitution of immunodeficiency. New Eng.J.Med.,298,222-226.

Zinkernagel,R.M. and Doherty,P.C. (1974). Restriction of in vitro T-cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature,248,701-702.

Zinkernagel,R.M. and Doherty,P.C. (1975). H-2 comptibility requirement for T cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for in H-2 K or H-2 D. J.Exp.Med.,141,1427-1436.

Zinkernagel,R.M., Callaghan,G.N., Althage,A., Cooper,S., Klein, P.A. and Klein,J. (1978). On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? J.Exp.Med.,147,882-896.

Thiery,J.-P., Brackenbury,R., Rutishauser,U. and Edelman,G.M. (1977). J.Biol.Chem.,252,6841.

van Heyningen,V., Guy,K., and Steel,C.M. (1982). Human MHC class II molecules as differentiation markers. Immunogenetics,16,459-469.

APPENDIX

This discussion essay is divided into two sections, dealing with chapters three and four of the thesis.

Discussion Pertaining to Chapter Three.

Any explanation of the polymorphism of the MHC must be based on the functions which it may control although it is theoretically possible that the polymorphism can be accounted for by assuming complete selective neutrality (Jones, 1980). In the absence of natural selection, differences in the level of polymorphism would have to be due to differences in mutation rates, implying that the MHC genes would have a correspondingly high mutation rate. This seems improbable.

By at least four mechanisms disease associations with the MHC may affect polymorphism (Bodmer, 1972): (i) molecular mimicry by one component of the pathogen of one of the MHC antigens leading to inability of the immune system to respond, (ii) interactions between MHC antigens and pathogen receptors, (iii) effects of MHC differences on efficacy with which pathogens are recognized and (iv) variations in the immune response to pathogens controlled by MHC-linked immune response genes. Each mechanism implies that the pathogen must adapt to the antigenic or immune response status of the host. If the immune response to the pathogen is improved, new varients may have a selective advantage until the pathogen responds.

It has also been suggested (Bodmer, 1972) that histocompatibility polymorphism (and the current complexity of the immune system) is a byproduct of the evolutionary need to develop a cell-cell recognition system for the differentiation and morphogenesis of multicellular

organisms. It is widely accepted that cell to cell recognition by like or associated cells may play an important part in differentiation. This recognition is likely to be mediated by cell surface structures of two types, differentiation antigens and their recognizers. Since the MHC contains a large number of genes and is so polymorphic, it can be viewed as a candidate for this function. In support of this it is known that Qa loci products are restricted to haemopoietic cells, that class II (Ia) antigen expression by thymic epithelial cells is important in thymocyte maturation (Jenkinson et al, 1982) and that melanoma cells, embryologically distinct from the lymphohaemopoietic system may also express class II antigens (van Heyningen et al, 1982). Additionally, the MHC has been linked with a multitude of seemingly unrelated phenomena for example levels of complement components (Demant et al, 1973), susceptibility to viral leukaemogenesis (Lilley, 1971) and liver cAMP levels (Meruelo and Edidin, 1974) all of which may result from interactions at the cell surface and this has lead to several groups studying the effect of MHC antigens on cell adhesion per se. Bartlett and Edidin (1978) found that the MHC played a significant role in the uptake of single fibroblasts by fibroblast monolayers although the single cells were not allowed to recover from trypsinization before being assayed. Curtis and Renshaw (1982) found that syngeneic combinations of lymphocytes and endothelial cells were less adhesive than allogeneic while Curtis and de Souza (1975) demonstrated that soluble histocompatibility antigens affect the adhesion of lymphocytes, fibroblasts and endothelial cells (Curtis, 1982).

How do MHC antigens compare with proteins known to be involved in cell adhesion ? In tissue distribution they differ greatly to the type of

cell adhesion molecule typified by NCAM and LCAM (neural and liver cell adhesion molecules) (Edwards, 1983) which are confined to one or several cell types and affect the adhesion of those cells only (Thiery et al, 1977, Bertolotti et al, 1980). The most widely documented protein involved in cell adhesion is fibronectin which is involved in a wide variety of cell-cell and cell-substrate adhesions (for review see Edwards, 1983). Fibronectin is essentially a component of the extracellular matrix, unlike MHC antigens which are generally thought of as membrane proteins although soluble fractions may be important in adhesion (Curtis, 1982). Thus MHC antigens do not fall easily into either category of cell adhesion protein, having similarities to both tissue-specific molecules and to fibronectin. Some workers, however, consider the specific cell-adhesion models to be incorrect. Curtis (1973), for example, has argued that there is little evidence for the occurence of specific cell-adhesion molecules but rather that these factors control a general mechanism of adhesion. This would be a better explaination of the MHC's role in cell adhesion, which is qualitative rather than quantitative.

The work presented in this chapter supports the theory that the MHC may play a role in cell adhesion. In every case studied, peritoneal exudate cells (PEC) adhere in significantly larger numbers to syngeneic than to allogeneic H-2D antigen-coated substrates. The assay system is a considerable simplification of previous assays in which two interacting cell surfaces had to be considered. Despite this, differences between allo- and syngeneic adhesion were not great (26 to 46%) suggesting that it may be of little importance in cell-cell adhesion. In support of this, no difference was seen between syngeneic and allogeneic

combinations of activated PEC. It had been hoped to study the effect of the soluble antigens on in vitro antibody synthesis to determine the biological effect of the antigens but technical difficulties prevented this. Curtis and coworkers (1979), however, have shown that small quantities markedly alter lymphocyte recirculation in vivo.

The results presented could be interpreted as suggesting that H-2D antigens may be responsible for the fine control of macrophage adhesion. The quantitative expression of class I antigens varies quite substantially from cell type to cell type (eg Nathenson and Cullen, 1974) and this could be the basis of differential adhesion. It would be of interest to examine macrophage adhesion to a range of concentrations of H-2D antigens, mimicking different cell types, to see if this would result in differential adhesion. It would also be worthwhile extending the range of cell types considered, especially cells outwith the immune system (since the MHC is known to be central to immune responses). If a variety of cell types was shown to be affected it could be taken as evidence for Bodmer's theory that the main function of the MHC is not the control of the immune system.

Macrophages are important cells in defence mechanisms and it is possible that the ability to discriminate between self and nonself is important to their protective function. The in vivo correlate of the allogeneic MHC antigens are antigens altered either by tumerogenic or parasitic agents. Variations in the ability of macrophages to adhere to non-self H-2D antigens may be reflecting differences in the ability of macrophages to adhere directly to targets in vivo. It is conceivable that for macrophages to adhere strongly to cells bearing altered MHC antigens in vivo, the cells must be opsonized with antibodies directed

against the altered antigen.

None of the work presented in this chapter addresses the nature of the adhesive interaction. It is not inconsistant with either a like-like interaction or a receptor-ligand interaction. The question could be approached by treating the cells with an anti-D antibody before the adhesion assay. This would be expected to alter results if a like-like interaction was responsible.

In summary, the role of H-2D antigens, if any, in cell adhesion could fall into two categories. It could be (possibly along with other MHC products) involved in the control of cell adhesion generally, the combinations and/or levels of expression lending the system its specificity, or it could be involved in the control of macrophage adhesion, particularly the response of macrophages to cells bearing altered MHC antigens. THe two possibilities are not mutually exclusive.

Discussion Pertaining to Chapter Four.

Current understanding of the role of MHC products in the immune response is not inconsistant with the view that these proteins are passsively anchored on the cell surface and are simply available for binding by receptors on other cells. It was the intention of this work to determine whether soluble products of the I region of the MHC could be attached to cells in an antigenically recgnizable form and, if so, to ascertain whether or not they could act as antigen presentation molecules. MHC antigens are transmembrane glycoproteins of which the bulk is found on the external surface but both class I and class II molcules have short cytoplasmic tails. While there appears to be no published work on the

function of the cytoplasmic sequences of class II antigens, much work has been done on class I molecules and is worth considering here. Firstly, sequence studies have shown that the cytoplasmic tail contains a phosphorylation site (Rothbard et al, 1980). Phosphorylation of serine groups by specific protein kinases is a well known method of regulating enzyme function and, by analogy, it has been suggested that the phosphorylation site on MHC antigens may be important in transmembrane signalling. It is possible that this mechanism may act through the interaction of the MHC antigens with actin (Koch and Smith, 1978) or another cytoplasmic component. Recently Murre and colleagues (1984) have conducted experiments to determine whether the cytoplasmic portions of the H-2 molecules of target cells are important in lymphocyte cytotoxicity. This was studied by constructing a novel H-2L molecule lacking the cytoplasmic section and introducing it into L cells where it is expressed at the surface. Since these cells were recognized by allospecific and virus-specific (where appropriately infected) cytotoxic cells, it was concluded that the cytoplasmic segment was not critical to the function of the molecule. As both classes of MHC molecule present foreign antigen, it is possible that the cytoplasmic tail of class II antigens is not essential for its function.

Cells capable of presenting antigen include cells of the monocyte/macrophage line, Langerhans cells of the skin, dendritic cells of lymphoid organs and mitogen-stimulated B lymphocytes (Chesnut et al, 1982). Possibly the most important of these physiologically are the macrophages because they can ingest and break down large particulate antigens and re-express partially degraded antigens on their surface. Macrohages fall into two subpopulations on the basis of Ia expression

and only Ia+ cells can function as antigen presenters. The exact relationship between the two populations is unclear; in vivo immunological stimulation results in recruitment of Ia+ cells from the blood rather than conversion of the resident population whereas in vitro the conversion of Ia- cells using the supernatant of activated spleen cells has been reported (Steeg et al, 1980). These cells were "immunocompetant" in as much as they were able to stimulate a mixed lymphocyte reaction. It is not known whether other changes take place simultaneously with the acquisiton of Ia positivity. One possibility is that the ability to produce interleukin-1, which is also essential for lymphocyte activation, is acquired with Ia expression. It has been shown, however, using lung macrophages, that Ia positivity does not correlate with secretion of IL-1 (Shelitto and Kaltreider, 1984).

In the experiments reported in this chapter, resident mouse peritoneal exudate cells, largely Ia-, were exposed overnight to supernatants of mitogen-activated spleen cells and Ia expression measured. These experiments showed that a significant proportion of cells became Ia positive. If an allogeneic combination was used, the resulting Ia expression was allogeneic to the macrophages, confirming that expression was exogeous rather than endogenous.

Attempts to determine whether the passive adsorption of Ia antigens onto cells conferred antigen-presenting ability were inconclusive, and were not reported in the thesis. The assay used was a T helper cell proliferation assay using primed, nylon wool-purified T lymphocytes and Ia preparation-treated peritoneal macrophages.

As an alternative, the effect of the Ia antigen preparation on mixed

lymphocyte reactions was assessed. This is a much less satisfactory assay because, although technically more simple to perform, its interpretation is much more difficult and it is not nearly such a good model of an in vivo immune response as the T helper cell proliferation assay. In these experiments soluble Ia antigens were added to MLR cultures, rather than one population being pretreated before culturing. The results showed that the addition of antigen syngeneic to responding cells resulted in diminution of the response, whereas addition of antigen allogeneic to the responding population brought about an increases in response in several assays carried out. In one instance shown (figure 12, page 12) addition of the Ia antigen had no significant effect. It would have been interesting in this case to have added stimulating cells at suboptimal concentrations, as any potentiating effect may have been obscured by using the stimulators at optimal concentrations.

It can be concluded from these experiments that soluble Ia antigens can attach to cells in an antigenically recognizable form and that they can then influence immune function. This is in agreement with the work of Murre et al (1984) eluded to earlier, which suggeted that MHC antigens may function without the need for specific cytoplasmic interactions. Unfortunately, no conclusion can be drawn on the question of whether or not passively acquired antigen can be used as a restricting element in the presentation of foreign antigen to T cells. The MLR experiments confirmed that adsorbed antigen was "immunocompetant" but it is not unreasonable to suggest that the requirements for antigen presentation may be somewhat different to those for inducing mixed lymphocyte reactions. What would be the implications of passively acquired Ia

antigens being able to present antigen ? In vivo, a significant amount of soluble antigen may be shed, especially by activated B lymphocytes at sites of immune responses. If these soluble Ia antigens were adsorbed by Ia-negative macrophages, it may confer on them the ability to co-operate in responses to large particulate antigens and, if picked up by other cell types then they may be able to cooperate in the response to soluble foreign antigens also adsorbed onto their surface. By these mechanisms, the capacity to develop a local, large scale immune response may be enhanced. It is likely that the adsorbed antigens would have a short half life in vivo so that they would act as short-term amplifiers of antigen presentation. The in vitro half life was not determined but is considerably shorter than seven days, after which Ia antigens are totally absent from the surface of PEC exposed to the soluble preparation.

In summary, exogenous Ia antigens can attach to cell surfaces are able to influence immune interactions.

References.

Bartlett,P.F. and Edidin,M. (1978). Effect of the H-2 gene complex on rates of fibroblast intercellular adhesion. J.Cell Biol.,77377-388.

Bertolotti,R., Rutishauser,U. and Edelman,G.M. (1980). Proc.Natl.Acad.Sci.USA,77,4831.

Bodmer,W.F. (1972). Evolutionary significance of the HLA system. Nature, 237,139-145.

Chesnut,R.W., Colon,S.M. and Grey,H.M. (1982). Antigen presentation by normal B cells, B cell tumours and macrophages: functional and biochemical comparison. J.Immunol.,128, 1764-1768.

Curtis,A.S.G. (1973). Cell adhesion. Prog.Biophys.Mol.Biol.,27,315-386.

Curtis,A.S.G. (1982). The genetic basis of cell behaviour in lymphocytes and other cells. In Bellairs,R., Curtis,A.S.G. and Dunn,G.A. (eds.) Cell Behaviour, Cambridge University Press, Cambridge.

Curtis,A.S.G. and de Souza,M. (1975). Lymphocyte interaction and positioning. Cell.Immunol.,19,282-297.

Curtis,A.S.G., Davies,M. and Wilkinson,P.C. (1979). New evidence of control mechanisms in lymphocyte traffic. In Muller-Reichholtz,W. and Muller-Hermalink,H.K. (eds.) Function and Structure of the Immune System., Plenum Press, London and New York.

Curtis,A.S.G. and Renshaw,R.M. (1982). Lymphocyte-endothelial interactions and histocompatibility restriction. In Nieuwenhuis, van den Broek and Hanna (eds.) In vivo Immunology, Plenum Press, London.

Demant,P., Capkova,J., Hinzovna,E. and Vorakova,B. (1973). The role of histocompatibility-2-linked Ss-Slp region in the control of mouse complement. Proc.Natl.Acad.Sci.USA,70863-866.

Edwards,J.G. (1983). The biochemistry of cell adhesion. Prog.Cell Surf.Sci.,13,125-196.

Jenkinson,E.S., van Ewijk,W. and Owen,J.J.T. (1981). Major histocompatibility complex expression on the epithelium of the developing thymus in normal and nude mice. J.Exp.Med.,153,280-292.

Jones,J.S. (1980). Nature 288,10.

Koch,G.L.E. and Smith,M.J. (1978). An association between actin and the major histocompatibility complex H-2. Nature,273, 274-278.

Manuelo,D. and Edidin,M. (1975). Association of mouse liver 3'5'cyclic monophosphate (cyclic AMP) with histocompatibility-2 genotype. Proc.Natl.Acad.Sci.USA,72,644-2648.

Murre,C., Reiss,C.S., Bernabeu,C., Cheu,L.B., Burakoff,S.J., and Seidmann,J.G. (1984). Construction, expression and recognition of an H-2 molecule lacking its carboxyl terminus. Nature,307,432-436.

Nathenson,S.G., Uehara,H. and Ewenstein,B.M. (1981). Primary structural analysis of the transplantation antigens of the murine H-2 major histocompatibility complex. Ann.Rev.Biochem.,50,1025-1052.

Rothbard,J.B., Hopp,T.P., Edelman,G.M. and Cunningham,B.A. (1980). Structure of the heavy chain of the H-2Kk histocompatibility antigen. Proc.Natl.Acad.Sci.USA,77,4239-4243.

Shellito,J.E. and Kaltreider,H.B. (1984). Heterogeneity in Ia expression and interleukin-2 production among rat alveolar macrophages. Am.Rev.Resp.Dis.,129(Suppl.),pageA5. (abstract).

Steeg,P.S., Moore,R.N. and Oppenheim,J.J. (1980). Regulation of murine macrophage Ia antigen expression by products of activated spleen cells. J.Exp.Med.,152,1734-1744.