THE ROLE OF THE MAJOR HISTOCOMPATIBILITY COMPLEX IN CELLULAR INTERACTIONS.

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CONTENTS.

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ABBREVIATIONS.

Ab	Antibody
B10	C57. Black 10 Sc/Sn congenic strains.
B cell	Bone - marrow derived lymphocyte which has not undergone thymic processing.
BHK	Baby Hamster Kidney fibroblasts.
C'	Complement.
^{14}C	Radioactive isotope of Carbon.
CMF	Calcium and magnesium free salt solution.
CML	Cell - mediated lympholysis.
CTL	Cytotoxic thymus derived lymphocyte.
DNA	Deoxyribonucleic Acid.
DNP	Dinitrophenyl.
ECGS	Endothelial Cell Growth Supplement.
EDTA	Ethylene Diamine Tetra - Acetic Acid.
EM	Electron Microscopy.
f1	Fraction 1 of conditioned medium, containing molecules with molecular weight up to 10 Kilodaltons.
F_1	First filial generation.
f2	Fraction of conditioned medium which predominantly contains molecules of molecular weight 10 - 35 Kilodaltons.
f3	Fraction of conditioned medium which predominanlty contains molecules exceeding 35 Kilodaltons molecular weight.
FCS	Foetal Calf Serum.

FDA	Fluorescein Diacetate.
FITC	Fluorescein Iso - thiocyanate. Isomer 1 on Celite.
gms.	Grams weight.
^{3}H	Tritium.
H-2	Histocompatibility-2 complex, the major histocompatibility complex of the mouse.
HIFCS	Heat - inactivated Foetal Calf Serum.
Ia	H-2 I region associated antigens.
Ig	Immunoglobulin.
IMF	Interaction - Modulation Factors.
Ir	Immune response.
ITS	Insulin / Transferrin / Selenium Medium.
K Daltons	Kilodaltons.
KLH	Keyhole Limpet Haemocyanin
LPS	Lipopolysaccharide
M	Molar.
µg	Micrograms.
mg	Milligram.
MLR	Mixed lymphocyte reaction.
mM	Millimolar.
N	Normal solution.
nm	Nanometer.
P.A.E.	Pig aortic endothelium.
PEC	Peritoneal Exudate Cell.
R.a.Hu.F.VIII	Rabbit Anti Human Factor VIII coagulation factor.
rpm	Revolutions per minute.
RPMI	Roswell Park Memorial Institute.

SAR-FITC	Sheep anti rabbit immunoglobulin, conjugated to Fluorescein Isothiocyanate.
SDS-PAGE	Sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis.
TEM	Transmission electron microscopy.
T cell	Thymus derived lymphocyte.
T-I.M.F.	Thymocyte - derived Interaction Modulation Factor.

SUMMARY.

1. Endothelial - like cells from murine lung cultures were shown to possess many endothelial - like properties. They appeared similar morphologically to Pig aortic endothelia, had similar intermediate filament proteins, stained positive for Factor VIII antigen, released large amounts of angiotensin converting enzyme, and transmission electron microscopy showed that the cells contained intracellular organelles indicative of an endothelial lineage.

2. Endothelial - like cells showed heterogeneity of H-2K^{k} expression. This was detected using complement - mediated cytotoxicty techniques in repeated exposure experiments and also by immunofluorescence staining of cell surface H-2K^{k}. This finding supports other work which demonstrated heterogeneity of H-2 expression in other tissue types. (Eskinazi et al, 1981).

4. Cells obtained from the peritoneal cavity adhere with greater spread areas but in reduced numbers when added to allogeneic H-2D fragment - coated substrates as compared to syngeneic H-2D coated substrates.

5. Peritoneal exudate cells spread out more and in increased numbers when pre-incubated in allogeneic, as opposed to syngeneic, conditioned medium. This effect was shown to be attributable to differences within the H-2K and / or I-A reion of the H-2 complex, since matching at these loci alone has no effect on spread area and counts as compared to fully syngeneic counterparts.

Mismatching of PEC's and conditioned media at H-2 K and / or I-A leads to increased counts and spread areas as compared to the effects of syngeneic conditioned media. These increases are significant at the P = 0.05% level of the standard t Test.

6. PEC's adsorb allogeneic I-A fragments from PEC or spleen cell conditioned medium onto their surfaces in an antigenically - recognisable form. Syngeneic I-A fragment binding was not detected by this technique. These results support a new model of cell interactions which has recently been proposed by Curtis (1983a).

7. Preliminary experiments have suggested that when endothelial - like cells of different H-2 types are mixed in aggregation cultures, they may sort out according to H-2 type such that one type surrounds the other in a typical "sphere - within - a sphere" arrangement synonymous with that seen in histotypic sorting out (Steinberg, 1964). This requires further investigation. It is unlikely that H-2 is directly involved in the cell interactions which occur during aggregation since aggregates of similar diameter were obtained with various mixtures of H-2 matched / mismatched cells present in the aggregating mixture. This finding supports previous observations on the collection of H-2 disparate cells to preformed aggregates (McClay and Gooding, 1978).

CHAPTER 1. INTRODUCTION.

1.INTRODUCTION

1.1. General.

One of the most fascinating aspects of animal development is the movement of cell types from one region in the embryo to another. Examples of this are seen during gastrulation, migration of cells from the neural crest and in the movement of precursor T lymphocytes into the thymus. Experimental systems which attempt to mimic the conditions within the embryo, in order to study these movements, have been difficult to devise. For this reason many workers tended to investigate the effects of transplanting parts of the embryo to differing positions or in differing orientations, an example of this being Twitty and Nui's work (1954). They investigated the repulsion between propigment cells by both transplantation and cell culturing techniques. In the former, they transplanted neural crest (from which pigment cells originate) to an area of the flank made pigment free. Propigment cells emerged radially from such a graft, but did not invade any areas already occupied by pigment cells. The cell culture methods involved the introduction of one or more propigment cells into fine capillary tubes. When a single cell was added, it remained more or less stationary in the tube, whereas two or more cells added together tended to move apart. Diffusible substance(s) produced by the cells appeared to be responsible for this "negative chemotaxis", though these were not identified. It is likely that negative chemotaxis plays an important role in some positioning systems, but other mechanisms must also be involved in both morphogenetic interactions and positioning mechanisms

operating throughout adult life, since it is unlikely that negative chemotaxis alone provides all the positional information required in these systems. We may ask the question: how does a lymphocyte leave the circulation and position itself within a lymph node containing many different cell types ? An answer to this question has been difficult to obtain, as the different cell types within the one tissue may interact with the lymphocyte in various ways. Attempts to study these interactions have therefore utilized simplified systems, i.e., interactions between two cell types only. These were facilitated by the development of *in vitro* reaggregation systems, though how closely these represent such interactions *in vivo* is questionable. A variety of different embryonic tissues can be dissociated to give single cell suspensions and if they are persuaded to reaggregate in appropriate conditions, they will often organise themselves so that the cells from different organs occupy different positions within the aggregate (Townes and Holtfreter, 1955; Moscona and Moscona, 1952). In a series of experiments using amphibian material (Townes and Holtfreter, 1955), the cell segregation and positioning was documented for various combinations of tissue slices as well as for the same combinations of disaggregated cells. Particular patterns emerged, which Townes and Holtfreter claimed, represented positioning and patterning arrangements which occur in the embryo. Further investigations extended to the segregation and positioning of aggregated cells of chick and mouse tissues. In 1964, Steinberg demonstrated a hierarchy of sorting out in combinations of chick tissues. For instance, cell type B was shown to segregate externally to A, whereas C would

segregate external to both A and B and so on. Many techniques were subsequently developed to study this sorting out phenomenon and despite their limitations, were interpreted as model systems of morphogenetic movement. However, cell combinations used in aggregation experiments were sometimes taken from tissues widely separated in vivo. Any sorting out abilities of these tissues seen in vitro may thus have limited, or no, morphogenetic relevance. The sorting out of one cell type with respect to another, such that one always segregates external to the other, implies that positional information is being generated within the aggregate. Two main hypotheses were put forward to explain the final positions adopted by mixtures of two or more reaggregated tissues. These were the Differential Adhesion Hypothesis (Steinberg, 1964) and mediation by cell products (I.M.F.'s - see Curtis and De Sousa, 1975). These theories will be dealt with more fully in the discussion.

Cell motility may be of importance in morphogenesis and in some cases the motility may result from decreased adhesiveness (Carter, 1965; Gail and Boone, 1972), though this does not necessarily follow (Curtis and Büültjens, 1973). Cell adhesion must frequently play a role in positioning both in the embryo and in the movement of cells in adult tissues. During cell movement, adhesive contacts must frequently be made and broken in order for a cell to exert a force against its cellular environment. Adhesion is thus likely to be regulated at the cellular level and show quantitative variation in that adhesiveness may be increased or decreased. Changes in adhesivity are known to occur during morphogenesis (see Bellairs, Curtis and

Sanders, 1978). Clearly then, the molecular mechanisms controlling cellular adhesive interactions form a central problem in these considerations. The search for such mechanisms is made more difficult by the fact that more than one adhesive mechanism is likely to be operating at any one time. It is probably an over-simplification to assume that a universal molecule will be found which is responsible for all morphogenetic movements. Instead, many mechanisms are likely to be interacting, to provide a fine degree of regulation of this process. One approach to the study of these is to identify the factors which block or enhance them. As yet, very few molecules have been found which can disrupt a morphogenetic interaction, if added or removed at the embryonic level. Retinoic acid has however been shown to possess similar properties to the polarizing region, which is involved in the development of the limb bud (Tickle et al, 1982). The polarizing region comprises a small group of cells which acts as a signalling region to specify the pattern of structures which develop across the anterior - posterior axis of the limb. The signal itself has been suggested to be a diffusible morphogen and can be mimicked by the application of retinoic acid bound to implanted inert beads (see Tickle et al, 1982). This was the first example of a well-defined spatial pattern of cellular differentiation being created by a chemical. It was concluded that the chemical probably changes positional values in a manner that closely mimics that of the normal signalling tissue. Evidence for similar chemical mimicry of pattern formation in other tissues has yet to be obtained.

In this thesis, consideration has been given to the role of the Major Histocompatibilty Complex products in cellular

interactions. Interest in the M.H.C. has grown such that it occupies a central position in immunobiology. The concepts developing in this area of research extend in their relevance beyond the immune system. It has now been established that M.H.C. encoded proteins are involved in cell-cell interactions in the immune response. The recognition of target cells by cytotoxic T cells, co-operation between T and B lymphocytes in antibody production and antigen presentation by macrophages to T lymphocytes are all regulated by M.H.C. encoded cell surface molecules. Evidence is accumulating that the M.H.C. may also play a role in non-immunological interactions as previously suggested by Bodmer (1972) and Edelman (1976) (see page 25). An appreciation of the nature and extent of M.H.C. involvement in these processes may be required for our further understanding of the mechanisms operating during morphogenesis.

1.2. Historical Aspects Of The Mouse H-2 Complex.

During the early years of this century, it was discovered that successful skin transplants could not be made between outbred mice. Similarly, passaging of tumour cells was only successful between genetically inbred individuals (see Little (1941), Snell (1953)). Rejection of transplants was subsequently shown to be an immunological phenomenon (Medawar, 1944). The serological demonstration of alloantibodies by P. A. Gorer (1938) were unified with histogenetical studies on the tissue antigens responsible for rapid graft rejection (Snell, 1948; Counce et al, 1956) and this resulted in the first descriptions of the Major Histocompatibility Complex (M.H.C.)

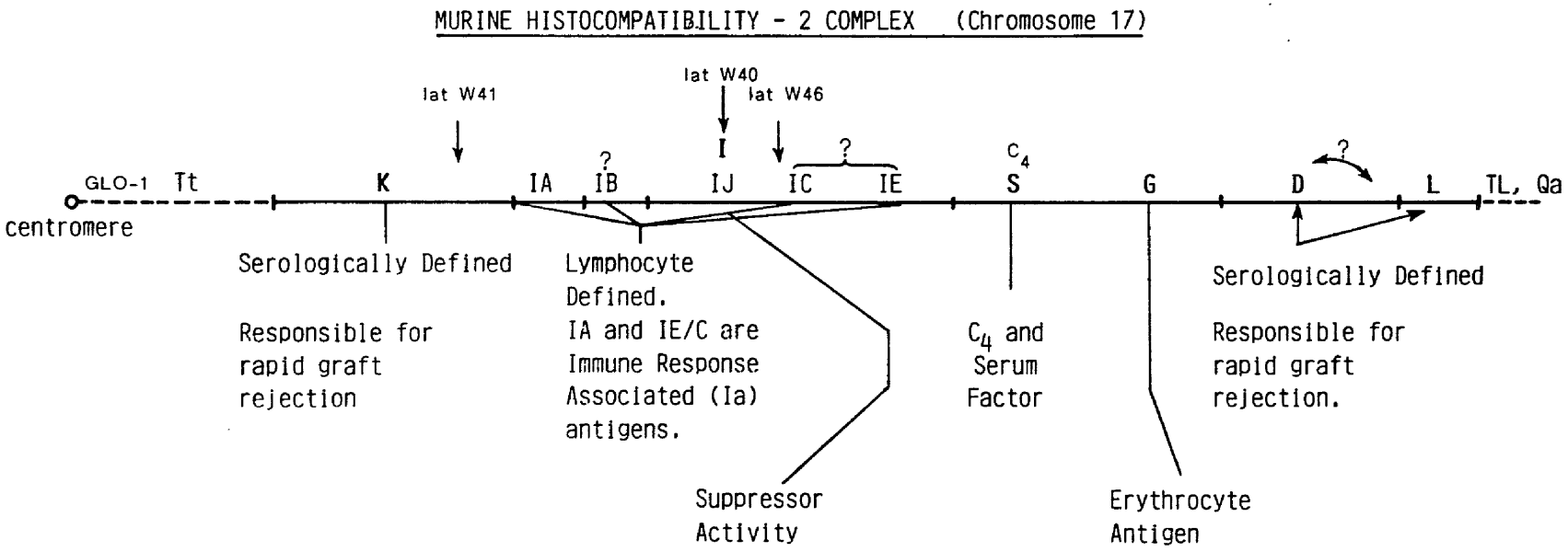
(Gorer et al, 1948, Counce et al, 1956, Mitchison 1954). All vertebrate species studied to date have an M.H.C. which dominates transplantation reactivity to foreign tissues (Klein 1975). Within some invertebrates, similar systems have also been identified (e.g. Schofield et al, 1982, Du Pasquier, 1974) though the genetics remains to be clarified in others (Curtis, Kerr and Knowlton, 1981).

1.3. Murine H-2 System: Structure and Function.

By far the most extensively characterised M.H.C.'s are those of the mouse, the H-2 Complex and man, the HL-A Complex. Both consist of several loci which are divided into three classes (Murphy, 1981). Within H-2, the K, D and L loci are named Class 1 proteins, originally defined by the serological activity of their respective products. The Class 2 (H-2I) loci lie between the K and D loci. Although these were originally defined by their presence and function on lymphocytes, some Class 2 proteins have subsequently been described on many tissues, where they may or may not play an immunological role (Nixon et al, 1982, Pober and Gimbrone, 1982). The Class 3 loci encode certain components of the complement system (Alper, 1981) and are generally considered non-H-2. (See fig 1).

Class 1 antigens are found in association with beta-2 microglobulin at the cell surface. They have a molecular weight of about 44,000 Daltons and are glycoproteins, having one or two carbohydrate sidechains. Studies using glycosidases (Nathenson and Muramatsu, 1971, Parham et al, 1977) and tunicamycin, an inhibitor of glycosylation (Ploegh et al, 1981) to generate

FIG. 1.


MURINE HISTOCOMPATIBILITY - 2 COMPLEX (Chromosome 17)
Iat W41
Iat W40
Iat W46
GLO-1
Tt
K
IA
IB
I
IJ
IC
IE
S
G
D
L
TL, Qa
centromere
Serologically Defined
Responsible for rapid graft rejection
Lymphocyte Defined.
IA and IE/C are Immune Response Associated (Ia) antigens.
Suppressor Activity
C4 and Serum Factor
Erythrocyte Antigen
Serologically Defined
Responsible for rapid graft rejection.

histocompatibility antigens lacking carbohydrate showed that the absence of carbohydrate had no effect on the antibody antigenicity or cytotoxic function of Class 1 antigens. Thus the alloantigenic determinants very likely reside in the polypeptide backbone of the heavy chain. Most of the Class 1 molecular complex is located extracellularly and this can be cleaved just exterior to the membrane by the use of the enzyme papain (Nathenson and Shimada, 1968). This technique has been crucial in the detailed structural analysis of these products, which have revealed structural analogies between H-2 and HL-A Class 1 heavy chains and immunoglobulin constant domains, as was suggested by Burnet (1970), Bodmer (1972), and Gally and Edelman (1972). Other work has shown that the Class 1 M.H.C. products may have a tetrameric structure and compact domains, which are strikingly similar to those in immunoglobulin molecules (Peterson et al, 1975: for discussion, see Greaves, 1975). Furthermore, beta - 2 microglobulin, which is associated with Class 1 products on the cell surface, has been shown to have considerable sequence homology with immunoglobulins (Cunningham et al, 1973: Raff, 1975). This may imply that a phylogenetic relationship exists between immunglobulin and products of the M.H.C. although in man there is no genetic linkage between the genes coding for HL-A, beta - 2 microglobulin and immunoglobulin. If such a phylogenetic relationship did exist, one could perhaps speculate on the involvement of M.H.C. products in antigen recognition. This issue has previously been raised with respect to the antigen - specific receptors employed by T cells. Whether M.H.C. products are involved in the recognition of antigen by T cells has still not

been resolved. In fact, recent evidence from anti - allotype and anti - idiotype studies has supported earlier speculation (eg. Gally and Edelman, 1972: Binz and Wigzell, 1976) that the antigen receptor on T cells may have a similar structure to immunoglobulin (Reinherz, 1983). Further evidence on the nature of the T cell receptor will be discussed elsewhere. Here, it is relevant to point out that M.H.C. products are known to play a central role in self - recognition in the immune system. Other workers have demonstrated that the M.H.C. may also be important in self - recognition between non - immune cells (see later section). The work presented in this thesis was partly designed to investigate these phenomena further.

As previously stated, the Class 2 products are encoded in the H-2I region. Structural analysis of this region is difficult as it exists at a high level of genetic complexity. With the use of recombinant strains of congenic mice, the region has been divided into at least five subregions; I-A, I-B, I-J, I-E, and I-C (Klein, 1979). The I-J region codes for products found on suppressor (Murphy et al, 1976) and some helper cells (Tada et al, 1978a, 1978b). (Interestingly, these products are also involved in the contact inhibition of movement seen in fibroblasts (Curtis and Rooney, 1979 - see page 29). It is as yet unclear whether I-E and I-C code for separate molecules, or indeed whether the I-B region exists at all. To date, only I-A and I-E/C encoded antigens have been structurally identified (see Murphy, 1981). In the mouse, the ability to respond to certain antigens is under the control of the Immune respose (Ir) genes which map to the I region (Möller, 1977). In fact, the vast majority of Ir genes have been localised

within the I-A subregion, as reviewed by Benacerraf and Katz (1975b) and Klein (1975). A small number of "complementing Ir genes" map in both the I-A and I-E/C subregions (Dorf, 1978) and finally, a few Ir gene - controlling reponses to selected antigens map in the I-B subregion (Lieberman et al, 1974: Ando and Fachet, 1977). Products of the I-A and I-E/C loci are thus implicated in the control of the immune response, which appears to involve cell - cell interactions as will be discussed later in the text. The I-E/C antigens are composed of two polypeptide chains of 33,000 and 25,000 Daltons, whereas I-A antigens form a complex of 58,000 Daltons. (For structural details, see Murphy, 1981; Orr, 1982). Determinants on I-A and I-E/C molecules manifest several important immunological properties, for instance CTL recognition (Simpson and Gordon, 1977; von Boehmer et al, 1977), serological activity, (Lafuse et al, 1980), CML stimulation (Peck et al, 1980) and Ir gene control. Clearly, the existence of several immunological properties or determinants does not necessitate that each be under the control of a separate locus (Fathman et al, 1981). It is possible that different subregions of the locus products are expressed on different cell types. For instance, T cells express a unique I-A subregion not found on B cells (Hayes and Hullett, 1982: Hiramatsu et al, 1982) and Jones et al (1978) have reported the expression of new Ia molecules as a consequence of co-operative activity of distinct I subregions. This implied heterogeneity of expression of Class 2 products on different subpopulations is interesting in the light of other experiments showing heterogeneity of Class 1 products, reported in this thesis and by others (Eskinazi et al, 1981). This will be discussed more

fully later.

In contrast to Class 1 antigens, the Class 2 antigens may have a more limited tissue distribution. Their function on macrophages seems to involve antigenic presentation, as will be discussed shortly. The larger alpha subunits (heavy chains) and the smaller beta subunits (light chains) associate non-covalently to form dimers, and these are displayed on the cell surface. Alpha and beta chains between species show a certain degree of homology, but in the mouse there is no homology between the alpha and beta chains from different subregions. Neither is it likely that the alpha chain is a proteolytic product of the beta chain. Furthermore, it is improbable that within a species the individual Class 2 loci developed from one another. The beta chain has been shown to be highly polymorphic, whereas the alpha chain is considerably less so. Thus, the beta chain is likely to be dominant in the determination of Class 2 alloantigenic specificity. As with the Class 1 antigens, it seems likely that the Class 2 antigens consist of a variable chain associated with a relatively invariant chain to form an alloantigenic complex. The protein portion of the I region products has been shown to contain the allodeterminants (Orr, 1982) and not the carbohydrates, although Higgins and Parish (1980) have reported that Ia determinants are also present on the carbohydrate portion of the glycolipids. For both classes of M.H.C. antigens the difficult task will be to determine which of the polymorphic residues contributes to the alloantigenic site and how the invariant chain is involved in determining alloantigenicity. (For further discussion, see Orr, 1982).

Having introduced the H-2 products it is now relevant to consider their involvement in cellular interactions. Most of this work has been performed using immunological systems, which are now well established. These will be considered here in detail.

1.4. Role Of H-2 In Immunological Interactions.

Both Class 1 and Class 2 products play a central role in immunological interactions. Although they are considered separately here, both types of product may be involved in the control of a particular event and similar restrictions are encountered in their control.

1.4.1. Immune Response (Ir) Genes And Restriction Phenomena.

McDevitt et al (1972) were the first to demonstrate that histocompatibility - linked Ir genes mapped to the H-2 I region. Since these initial observations, both cellular and humoral responses to many types of antigens in several different species have been shown to be under antigen - specific Histocompatibility Complex - linked Ir gene control (Benacerraf and McDevitt, 1972). Immune responses that are regulated by H-2 -linked Ir genes require the presence of thymus - derived lymphocytes. These T cells, which bear the Ly-1 antigen, recognise thymus - dependant antigens in conjunction with autologous I region products. Once this interaction has occured, the T cells can provide the essential helper signal for differentiation of B cells into antibody - producing cells (Katz and Benacerraf, 1972). (It may be important to note that to date, M.H.C. - linked Ir gene control of a thymus - independant response has never been observed.) In many of the systems investigated, it was speculated that the

interactions of helper T lymphocytes with B lymphocytes were governed by genes that map in the same I-A and I-C subregions that control macrophage - T cell interactions (Benacerraf and Katz, 1974). These observations were originally made by Kindred and Shreffer (1972) and by Katz et al (1973) close to the time when the role of I - region products in macrophage - T cell interactions were discovered. However, other workers have recently reported that T - B cell and macrophage - T cell interactions may not be controlled by the same I region genes (Nagy and Klein). This work will be discussed below.

The first demonstration that T cell - mediated immune responses were also regulated by Ir genes involved the demonstration that Dinitrophenyl - Poly-L-Lysine (DNP-PLL) nonresponder guinea pigs failed to produce delayed - type - hypersensitivity (DTH) responses to this antigen (Green et al, 1966, 1967). In many Ir gene systems, nonresponder animals were proved to lack carrier - specific helper activity (Benacerraf and McDevitt, 1972). M.H.C. - linked Ir genes specific for minor histocompatibility antigens such as H-Y were subsequently shown to control additional cell - mediated immune responses, including graft rejection and the generation of cytotoxic T cells (Simpson and Gordon, 1977; von Boehmer et al, 1977). Furthermore, T cell proliferation has been shown to be under the control of H-2 linked Ir genes, the responder / nonresponder pattern being very similar to that described for antibody - forming capacity (Bluestein et al, 1971: Shevach et al, 1972). Since helper T cells participate in both these types of interaction, the simplest explanation to relate these two phenomena is that Ir gene control operates at the

level of stimulation of T cells. The evidence in support of this explanation will now be considered in detail.

Early work by Shevach and Rosenthal (1973) and Schwartz et al (1979), demonstrated the expression of Ir genes on macrophages. Their role is to produce, together with antigen, an immunogenic stimulus capable of activating T cell clones specifically. In this respect, this model assigns to the Ir gene product the same role in the generation of specific helper T cells that is played by the K and D gene products in the generation of the cytotoxic T cell response (see next section). Furthermore, it is now widely believed that certain Ir genes are, in fact, equivalent to Ia antigens on macrophages and some workers have suggested that the lack of appropriate Ia prevents antigenic presentation and the stimulation of "positive" effector T cells such as helper, proliferative, or DTH - inducing cells (Benacerraf, 1978). Furthermore, Schwartz et al have demonstrated that <u>in vitro</u> antigen - induced proliferative responses of T cells from primed (responder x nonresponder) F_1 can be inhibited only by specific anti - Ia antisera against the alleles of the responder parent.

This leads us to a subject of recent debate, namely, whether Ir genes function at the level of the macrophage or the T cell. One group have produced results which suggest that animals of nonresponder strains have an intrinsic defect in Ir gene product function on their macrophages (Shevach and Rosenthal, 1973, Shevach et al, 1973, Shevach, 1976, Rosenthal et al, 1977, Rosenthal, 1978). However, Nagy and Klein (1981) have argued that controlling Ir genes are expressed at the level of the T cell and

those Ir genes which are expressed on macrophages do not control the interaction. Furthermore, these workers showed that macrophages are able to present antigen to M.H.C. disparate T cells if the two are cultured in vitro. They concluded that there is no evidence for the same restriction as is seen in cytotoxic T cell - T helper cell interactions. This seems to be at variance with earlier work (Rosenthal and Shevach, 1978). They argued that the combination of Ir genes and antigen on the macrophage surface in nonresponder strains may look too similar to self M.H.C. and therefore not be recognised due to depletion of T cell clones during self education (Schwartz, 1976, 1978, 1979). Further work is being done to resolve the discrepancies (see Nagy and Klein, 1981, Shevach, 1982, Rosenthal, 1982). However, one point raised by Nagy and Klein is the suggestion that macrophages can collaborate with allogeneic T cells. Earlier work by Sprent (1978) has demonstrated that macrophages are unable to activate H-2 I allogeneic T cells, and similarly, both primed and unprimed T cells failed to collaborate with allogeneic B cells (see Benacerraf, 1981 for full discussion). The work of Kappler and Marrack (1977) demonstrated that antigen - specific primed helper F_1 T cells could be functionally selected on antigen - pulsed parental macrophage monolayers so as to provide helper activity to the helper B cells of the same haplotype as the macrophage monolayer used in the selection. This implies that the same restriction is operating in the M.H.C - restriction of macrophage - T cell interactions and T - B cell interactions. However, Singer et al (1979) have recently investigated the anti - hapten response to Trinitrophenyl - Keyhole Limpet Haemocyanin (TNP-KLH) and have

assessed the helper T cell recognition of H-2 determinants on macrophages and B cells independantly. Although they confirmed the requirement for helper T cell recognition of MHC determinants on macrophages proposed by Rosenthal and Shevach (see above), they failed to find corresponding restriction in the interaction of T cells with B cells. Other groups have similarly reported that T - B interactions are not always M.H.C. restricted, whereas T cell - macrophage interactions are (Hodes et al, 1983; Julius et al, 1982; Ratcliffe et al, 1982). Discrepancies in results on the roles of M.H.C. in B - T interactions may depend on the source of the macrophages used to activate the helper T cells and work is in progress to resolve these differences.

Other models exist which are not entirely consistent with a defect in antigenic presentation in nonreponder animals. For instance, work done on GAT, (a synthetic polymer of glutamic acid, alanine, and tyrosine) has shown that a) cyclophosphamide - treated (Germain and Benacerraf, 1979) or anti I-J antiserum treated (Pierres et al, 1978) nonresponder mice give primary GAT responses in vivo and b) nonresponder macrophages can present GAT for primary in vitro responses by responder strain lymphocytes (Pierce et al, 1976). Thus, for GAT, reduced ability of nonresponder macrophages to trigger appropriate helper cells may result in the predominance of an in vivo suppressor T cell response. This may occur because of direct recognition of soluble antigen by suppressor cells (Pierres and Germain, 1978) or by presentation of GAT on cell membranes, perhaps without the involvement of M.H.C. gene products. However, there is little evidence that suppressor T cell responses account for

nonresponsiveness to other antigens under Ir gene control, but whether the nonresponsive status results from a defect in macrophage presentation or in a lack of appropriate T cells is as yet unresolved.

Having discussed T - B and macrophage - T cell interactions, it is now relevant to discuss a T - T interaction system, namely cell - mediated cytotoxicity. Although a great deal of work has been done on this interaction mechanism, the events taking place at the molecular level during CTL killing are still open to speculation. The various hypotheses which attempt to explain the interaction mechanism will be discussed in some detail.

1.4.2. Cytotoxic T Lymphocytes And Restriction Phenomena.

Model systems to study cell - mediated cytotoxicity in vitro were developed during the late 1960s (Perlmann and Holm, 1969). Lysis of allogeneic target cells in vitro was found to be directed at products of either the H-2K, -D, or -L region (Abassi et al, 1973, Nabholz et al, 1974) since targets must be matched at these loci with stimulator cells if lysis is to occur. Studies have shown that alloantibodies directed at target cell H-2 (Möller, 1965; Brunner et al, 1968; Canty and Wünderlich, 1970; Nabholz et al, 1974; Faanes and Choi, 1974) and monoclonal antibodies against H-2K or -D region products (Lindahl and Lemke, 1979; Epstein et al, 1980) block lysis of target cells specifically. These studies could not rule out completely a role for molecules controlled by loci very closely linked to H-2K or -D. However, results from studies of H-2 mutant mice also provide

very strong evidence to support the idea of H-2K or -D involvement in recognition. Mutations in H-2 (particularly H-2K^{b}) have been found and CTL recognition of these mutants mimics allorecognition (Klein, 1978; Nairn et al, 1980). The finding that H-2K, -D, and -L products are the major target cell antigens recognised by allogeneic CTL's is in agreement with <u>in vivo</u> results (Cerottini and Brunner, 1974; Shreffler and David, 1975). The presence of specific receptors on CTL's was inferred by the finding that cytolytic activity could be absorbed out on monolayers of allogeneic cells (Brøndz, 1968; Golstein et al, 1971; Berke and Levey,1972). Martz has extensively described the sequence of events which lead to cytolysis of specific target cells (Martz, 1977, 1980; Martz and Benacerraf, 1973, 1976). Briefly, responder cells bind specifically to a target cell via a magnesium ion - dependent process which is then followed by the calcium ion - dependent programming of the target cell for lysis. The CTL remains unaffected by this encounter and can then move away in search of another target cell. However, as a result of this lethal hit, the target cell lyses even though it may not have maintained contact with the CTL effector cell. There have been numerous attempts to dissect this sequence of events, particularly with the use of blocking agents. These experiments have been discussed elsewhere (see for instance Todd et al, 1975; Linna et al, 1978; Martz, 1980).

Of more relevance here is the work reported by Zinkernagel and Doherty, (1974a, 1974b). They showed that after viral modification of targets, CTL effectors are only able to recognise foreign antigen when it is presented in the context of

self H-2K,-D, or -L molecules. Independently, Shearer (1974) found that CTL's can only lyse TNP - modified targets which carry the same K, D, or L products as the responder population. Analogous results have also been obtained for CTL's generated against minor histocompatibility loci (Bevan, 1975a; Gordon et al, 1975). These phenomena became known as the M.H.C. Restriction of CTL killing. Furthermore, it was discovered that the antigen (or modified-self protein) must be expressed on the same cell membrane as the self - K, -D, or -L molecule for CTL - mediated lysis to take place (Bevan, 1975b; Swierkosz et al, 1977; Watt and Gooding, 1980). The restriction of peripheral CTL's to self K, D, or L products is imposed during their intrathymic maturation phase independent of the presence of the extraneous antigen. It has been pointed out that other T cells which are non-restricted are not necessarily removed in the intrathymic maturation phase. Their presence is still detectable in the periphery, which led to the conclusion that many but not all T cells show restriction (Stockinger et al, 1981). However, H-2 restriction in CTL - mediated lysis has now been demonstrated in a large number of systems (Zinkernagel and Doherty, 1979). A number of workers have shown that this restriction of CTL's against virally- or chemically-altered targets is not dependent on the H-2 type of the CTL precursors themselves. Instead, it is determined by the H-2 type of the thymic environment in which they mature (eg. see Zinkernagel et al, 1978; Fink and Bevan, 1981). Recent work has however suggested that CTL's are capable of differentiation along both an intra-thymic and an extra-thymic differentiation pathway, such that they may not be fully restricted by the thymic environment (Singer, Hathcock and

Hodes, 1982). This may provide supportive evidence for the presence of non-restricted T cells described by Stockinger et al (1981). The results of Singers group were in contradiction to previous results (Zinkernagel et al, 1980; Kindred, 1978) though Singers group used only those spleen cells which did not adhere to nylon and used completely inbred strains (unlike Zinkernagel's group). This may account for the discrepancies between results.

Several laboratories (Nabholz et al, 1975: Wagner et al, 1975: Klein et al, 1976) have recently reported that the I region also codes for CTL target antigens. CTL's have been generated after a primary in vitro culture between strains differing only at the I region (eg; A.TH anti A.TL, or AQR anti B10.6R). Klein et al (1976) have generated I region - specific CTL's by first priming with skin grafts differing at the I region and then restimulating in vitro with I region - different lymphoid cells. By stimulating with a whole I region difference and then testing on targets that share only certain I subregions with the stimulators, it appears that the major I - region target antigens are encoded in the I-A subregion (Wagner et al, 1975). Furthermore, Klein et al (1977) demonstrated that I - region antigens did exist as independent transplantation loci. They found that LPS - stimulated blast targets (LPS is a B cell mitogen used to stimulate spleen lymphoblasts) could be lysed only if they shared the I region with the stimulator and there was no requirement for sharing of the H-2K or H-2D antigens. Billings et al (1977) also found that antisera specific for the I region of the target could inhibit lysis, but antisera to the H-2D region products had no effect on target cell lysis. Clearly, the role of H-2I region products in

CTL systems requires further investigation.

At this point, I must digress to examine the explanations which have been put forward to account for H-2 restriction phenomena in CTL systems (Doherty et al, 1976). Analysis of antigen recognition by cytotoxic T lymphocytes and other H-2 restricted T cells has yielded results which are generally explained by one of two models. These are-;

1.4.2.1. Altered Self Hypothesis.

According to this hypothesis, a single receptor on the cytotoxic T cell binds to a complex on the target cell surface. This complex is formed by the association between antigen x and the appropriate H-2 molecule (K, D, or L - see page 18). The complex recognised is generally thought to be a unique structure formed by this association. Evidence in support of this theory has been extensively reviewed elsewhere (see for eg. Matzinger, (1981), Pasternack and Eisen, (1982)). Here a brief summary of this is relevant to the text. Hybridomas formed from two CTL's recognising either antigen x + H-2a, or antigen y + H-2b, can respond only to these combinations of antigen and specific H-2 (Kappler et al, 1981). No response is obtained against y + H-2a or x + H-2b. The interpretation is that CTL's can only recognise the same complex of H-2 and antigen with which they were stimulated because these exist as a complex or "neoantigen", with specificities not found on either the H-2 or antigen singly (Kappler et al, 1981). Co-precipitation of H-2 and tumour-associated antigens in detergent extracts, (Callahan and Allison, 1978; Kvist et al, 1978; Honeycutt and Gooding, 1980) and co-

capping of viral- or tumour-associated antigens with H-2 (Schrader et al, 1975; Zarling et al, 1978; Senik and Néauport-Sautès, 1979) have also been reported. Friend leukaemia virus particles selectively incorporate some host H-2 antigens. The antigens incorporated correlate with the H-2K, or -D to which the virus specific CTL's are restricted (Bubbers and Lilly, 1977; Blank and Lilly, 1977; Bubbers et al, 1978). CTL's against chemically-or virally-modified self H-2K or -D targets sometimes cross-react with modified allogeneic targets (see Pasternack and Eisen, 1982). Explanations for all the above - mentioned results have utilised the the Single Receptor (Modified or Altered Self, or Interaction Antigen) model.

1.4.2.2. Dual Recognition Hypothesis.

This hypothesis proposes that the CTL's and their precursors have one receptor for antigen and one for the restricting self H-2 molecule. Both of these receptors must be bound to specific ligand on the target cell surface before effective CTL and target cell conjugates can form. The failure of H-Y antigens to co-cap with either H-2 (Geib et al, 1977) or HL-A (Fellous et al, 1978) provides evidence in support of a Dual Recognition model for CTL killing of H-Y targets. Recently, Müller (1981) has shown that after CTL or antibody binding of H-Y antigens, H-2D products are brought into closer proximity. The secondary binding of H-2D to the CTL receptor is of much higher affinity and facilitates CTL-mediated lysis. If cytochalasin B is added to effector CTL's before the assay, lysis is inhibited. This treatment is known to disrupt actin filament organisation and it

has been postulated that this may inhibit formation of bonds between the H-Y receptor and the H-2D receptor (Simpson and Gordon, 1977; Matsunga and Ohno, 1980). Further evidence for this hypothesis was demonstrated by the failure of VSV antigen and H-2 to redistribute reciprocally (Geiger et al, 1979). Anti - VSV antibody selectively redistributed the H-2 molecule to which killing was restricted, but VSV antigen was not redistributed by anti H-2. This argues strongly against the Altered Self hypothesis, unless the association of H-2 and antigen is of a dynamic nature as proposed by Matzinger (1981).

To date, neither the Altered Self nor the Dual Recognition hypothesis has been eliminated, and some lines of evidence favour very strongly one or the other as shown above. The relationship between the H-2 restricted and the allogeneic CTL's remains to be clarified. Supporters of the Dual Recognition hypothesis view allogeneic killing as a special case (see Matzinger, (1981) for comments). It has been suggested that the CTL's responsible for these two types of interaction arise as a result of different selection processes (Dröge, 1981). In humans, two different CTL sub-populations have been recognised on the basis of different cell surface molecules. One carries the T3 and T4 glycoproteins and is directed against antigen in the context of Class 2 molecules. The other carries T8 and T3 glycoproteins and recognises antigen in the context of Class 1 molecules. Antibody blocking studies have suggested that T4 and T8 bind the Class 2 or Class 1 M.H.C. respectively on the target cells, whereas T3 in association with another receptor (Ti_n) binds the antigen (Reinherz et al, 1983). The Ti_n receptor is thought to be of

constant binding specificity within a CTL clone but variable between clones and thus is responsible for a wide range of antigenic specificities within the CTL repertoire (see Reinherz et al, 1983 for review). A similar glycoprotein distribution on murine CTL's has not yet been categorised. Considering the similarities between M.H.C. and immune systems of mice and humans, it seems likely that an analogous system will be identified.

In conclusion, it is possible that CTL, T - B, and macrophage - T cell systems reflect similar control systems, as previously proposed by Zinkernagel (1976). The mechanism whereby H-2 restriction operates is still open to speculation. Katz has suggested that cell interaction (C.I.) molecules interact on the surface membranes between communicating cells, either by homology or complementarity. Homologous interactions require that the C.I. molecules on the respective cells are products of identical genes, whereas complementarity implies the reaction of a T cell receptor entity with a C.I. molecule on the corresponding B cell, T cell or macrophage, the respective molecules (i.e. the receptor and C.I. site) being products of distinct genes. These two interaction mechanisms could occur in different situations, for instance, syngeneic interactions occur via homology while allogeneic interactions could occur via complementarity. (For full discussion, see Katz, 1977a, 1977b, 1978). The importance of H-2 restriction in CTL systems is particularly evident in defence mechanisms. The specificity for antigen + self H-2 rather than antigen alone means that a CTL will obliterate those cells which are producing virions. Obviously, this is a more efficient way of controlling viral infections than by inactivating individual

extracellular virions. H-2 restriction may also provide one explanation for the extraordinary polymorphism of the Major Histocompatibility Complex genes (Snell et al, 1976). Although the molecular mechanisms operating remain obscure, the implications of H-2 restriction and polymorphism have already led to a great deal of speculation on the role of these products in cellular interactions reaching beyond the immune system, into the realms of cellular and developmental biology. An outline of these theories, together with evidence in support of them, will now be considered.

1.5. Role Of H-2 In Non-Immunological Interactions.

In 1972, Bodmer suggested that the prime function of the M.H.C. might be to control cell-cell interactions within an organism. Although he did not present any experimental evidence to support this theory, his arguments were based on three main observations. Firstly, cell positioning within organisms is probably a result of various types of intercellular recognition. Clearly, this requires a system with multiple instructions which could be provided by the M.H.C. due to its highly polymorphic nature. Secondly, as we have just seen, many immunological interactions are M.H.C. or multilocular controlled. Finally, Bodmer argued that other explanations for the major role of the M.H.C., ie. in the recognition of self and non - self, appear somewhat inadequate. In subsequent years, it has become clear that the M.H.C. does play a major role in determining whether a range of cell interactions would take place. The evidence for this statement will now be considered.

1.5.1. Evidence That H-2 Systems May Affect Cell Interactions.

A series of experiments investigating the role of H-2 products in cellular interactions have employed the technique of monolayer collection, developed for rather different purposes by Walther et al (1973). Cell suspensions are usually prepared by dissociation with trypsin and are allowed to settle onto a monolayer of living cells. After a short period (usually up to 30 minutes) non-adhering cells are washed off and the proportion of cells bound from the suspension is measured. Other workers have reported that cells may take up to several hours to recover their

specificity of adhesion after trypsinisation, but results on specific adhesiveness have nevertheless been obtained by this method. For instance, in 1978, Bartlett and Edidin examined the adhesion of syngeneic and allogeneic fibroblasts and found evidence for H-2 restriction of adhesion. They investigated several strain combinations and found that allogeneic cells sometimes show slightly reduced adhesion when compared to syngeneic combinations. Their interpretation of these results was to suggest that the monolayer controlled the selectivity of adhesion, rather than both the suspended cells and the monolayer. In their results, the differences between syngeneic and allogeneic combinations were not very marked, though combinations extensively mismatched at H-2 tended to have the lowest adhesion rates. Zeleny et al (1978) used the same method to demonstrate H-2 restricted adhesion in bone marrow and lymph node cells. However, the cell types involved were not precisely identified and the effect on adhesion was a small diminution between strains B10.A(2R) and B10.A(5R) which have the haplotypes kkkkkdddb and bbbkkdddd respectively. Although they could not identify which loci were involved in the restriction, their results suggested that IJ, IE, IC, S and G loci were not involved. Similarly, Bartlett and Edidin did not identify the loci involved in fibroblast restriction, though it would have been of interest to compare these systems.

It is of importance to consider here that the restriction phenomenon is not confined to the vertebrates. Indeed, a great deal of work has been performed on freshwater and marine sponges, which both demonstrate restriction for strain types. Curtis and Van de Vyver (1971) investigated the non-coalescence

between different strains of Ephydatia fluviatilis. They found that each strain type produced diffusible molecules which specifically reduced the adhesion of cells of other strains and pointed out that this was an example of natural histocompatibility. They concluded that although the factors were themselves specific, the effect of a reduction in adhesion was non-specific in that the cells now had their adhesiveness to any other surface lowered. This was the first demonstration of involvement of diffusible molecules in the reduction of adhesion of another cell type and subsequently these factors became known as Interaction-Modulation Factors (I.M.F.'s). Later, using the marine sponge Hymeniacidon, Curtis (1979a, 1979b) managed to demonstrate that production of and reaction to I.M.F.' correlated precisely with graft rejection and non-coalescence between strains of this species. It is reasonable to assume that I.M.F.'s play an important role in the maintenance of integrity within a strain type and it is interesting that similar methods exist for maintaining the integrity of tissue types within both embryonic and adult tissues of higher organisms, which are discussed below. These experiments pose some interesting points on the maintenance of such mechanisms through evolution.

In 1974, Curtis found evidence that similar factors to those described in sponge systems were released by embryonic liver and neural retinal cells. Low molecular weight I.M.F.'s were secreted into the culture supernatant by actively-growing cells and decreased the adhesiveness of the opposite cell type. Addition of I.M.F.'s to a 1:1 mixture of cell types biased aggregation such that in the presence of neural retinal I.M.F., aggregates became

rich in neural retinal cells, while in the presence of liver I.M.F., they became rich in liver cells. In the presence of both factors, aggregates, though small, became random in composition. I.M.F.'s could also alter the positioning of cells if added to the aggregating mixture during sorting out. The evaluation of a molecule in cell positioning mechanisms requires that it is capable of such a function. In later work, Curtis and de Sousa (1975) discovered a system, identical in many respects to that in sponges and embryonic cells, operating in lymphocyte behaviour both in vitro and in vivo. They prepared B and T lymphocyte suspensions from congenic mouse strains and looked at the specificity of adhesion of these cells. If mixed B and T cell suspensions were well washed, no specificity of adhesion was seen either in the composition of aggregates formed, or in the kinetic assay of specificity of adhesion. However, if the medium was preconditioned with one or both cell types, apparent specificity of adhesion was seen. As in the embryonic and sponge systems, conditioned media reduced adhesiveness of the other type of cell (see Curtis, 1982 for full review). Additionally, they found that I.M.F.'s could reduce the adhesiveness of the same cell type when they differed at particular H-2 loci. They demonstrated that H-2D was involved in the restriction of T-I.M.F. and H-2K or -I-A were involved in the restriction of B-I.M.F. in that they could only act on target cells matched at these loci. Furthermore, T-I.M.F.'s were shown to be identical in nature to the papain-cleaved fraction of the H-2D product (Curtis and Davies, 1981). The questions which arise from this work relate to the ability of these factors to disrupt or promote in vivo functions. Some work

was carried out to investigate such properties. The ability of I.M.F.'s to alter lymphocyte homing mechanisms if preincubated with injected lymphocytes, and to radically alter in vivo cell positioning if injected into mice, do in fact suggest that I.M.F.'s could play a major role in lymphocyte interactions and positioning mechanisms in vivo. Further implications of these findings will be discussed later.

The evidence discussed above implies that there is allogeneic inhibition of cell adhesion. It is relevant here to ask whether or not cell spreading is similarly affected by the proximity of allogeneic cells or factors. Medawar (1978) was probably the first to suggest that the M.H.C. may be involved in the contact inhibition of movement shown by fibroblasts and epithelia. This theory was tested in 1979 by Curtis and Rooney. By confronting kidney epithelial cell outgrowths from different aggregates, they showed that contact inhibition, as assessed by the overlap index, was more marked between allogeneic than between syngeneic combinations and most particularly when the genetic mismatch was at K, or I-A and D. The idea that contact inhibition of movement is involved in organogenesis and is itself shown here to be under M.H.C. control, places the M.H.C. in a central role in this process.

Although the above investigations have all indicated a reduction in adhesiveness between allogeneic cell types, not all combinations of tissue types fall into this classification. For instance, it has been shown that lymphocytes adhere more readily to allogeneic capillary endothelial cell monolayers (Curtis and Renshaw, 1982), in a classical collecting lawn - type assay

described previously (Walther et al, 1973). Considering the function of lymphocytes in immunological responses, it is perhaps not suprising that such cells would adhere more readily to allogeneic rather than syngeneic cell types. Discrepancies in such results have however appeared in the literature. Haston (1979) added lymphocytes or thymocytes to cultures of lymph node cells (which include lymphocytes, reticular cells, endothelial cells etc.) but found no evidence for differences in the binding of syngeneic or allogeneic combinations. Explanations for these discepancies may be found in the differences due to cell types used in the monolayer. and also in the fact that Haston's cultures were well washed, a procedure known to reduce specificity (Curtis, 1974). Furthermore, other experimental evidence has been produced which argues against H-2 involvement in some cellular interactions. McClay and Gooding (1978) used embryonic liver and brain from C3H and B10 strains to compare tissue type specificity with strain type specificity. By labelling one cell suspension with ^{3}H leucine and another with ^{14}C leucine, they compared the collection of cells to aggregates using various tissue and strain combinations. Quantitative comparisons were made between collections by calculating the Recognition Index for all combinations used. They concluded that although the cells showed tissue specificity, no allogeneic effect was seen in these <u>in vitro</u> models. They argued that the M.H.C. is unlikely to be involved in tissue interactions <u>in vivo</u> for two main reasons. Firstly, the highly polymorphic nature of the complex suggests that a heterozygotic outbred individual would express two alleles of a given locus. They argued that if H-2 alleles were used for

recognition, the genetically variable portion of the molecues must not be important in recognition events. Otherwise, morphogenetic events would be further complicated by a need to recognise individual differences. Secondly, they investigated the differences in H-2 expression of liver cells after dissociation either by mechanical methods or with trypsin using polyclonal anti-H-2 antibodies and complement. They found that trypsinised and non-trypsinsied cells showed no differences in their ability to be lysed by these methods. They interpreted these findings in that H-2 specificity is relatively insensitive to trypsin, whereas adhesion events are highly trypsin sensitive and thus H-2 products cannot be directly responsible for the molecular interactions taking place during cell adhesion. However, this work does not rule out an indirect role for these products.

To summarise the evidence mentioned above, it may be that H-2 products affect cell interactions by altering the level of adhesion of their target cells but are not themselves the molecules taking part in the adhesive mechanisms. Within the CTL system, it is possible that H-2 regulates the binding of viral or allogeneic antigen without being as directly involved as predicted by the Altered Self and Dual Receptor hypotheses. The central question arising from these considerations is; how do syngeneic tissues within one organism differ sufficiently to separate and position themselves in different places? One explanation of this phenomenon is that the display of M.H.C. antigens varies between different tissues such that pairs of cell types, one lacking and one expressing the products of a certain locus will interact in a different manner from those sharing its expression. In other

words, differential expression of H-2 antigens may occur. Perhaps cells express only some of the H-2 antigen set present in the genome. Within the immune system, evidence for this has already been obtained (see Hayes and Hullett, 1982). Evidence for differential expression of H-2 products in embryonic tissues has been obtained by the following observations. Jenkinson and Searle (1979) looked at Ia expression on the embryo and placenta using immunoperoxidase techniques and a mixed haemabsorption assay. They found that Ia antigens were absent from preselected pre- and post-implantation embryonic and trophoblastic tissues and from the trophoblast of the definitive placenta. In contrast, cells derived from the peri-placental maternal tissues were Ia - positive. Ostrand-Rosenberg et al (1977) looked at the expression of H-2 antigens on cell lines from four day old mouse blastocysts. These were derived from $H\text{-}2^{s}$ x $H\text{-}2^{q}$ crosses. Using H-2 typing sera with direct microcytotoxicity and indirect absorption studies, they demonstrated that at least one public or private specificity of each D^{q}, K^{q}, D^{s} and K^{s} region was present on these cells. However, they concluded that they could only detect partial expression of H-2 specificities and discussed the possibility that incomplete H-2 molecules could be present on the cell surface, which could have effects on cell positioning during ontogenesis. Three theories were put forward to explain these results-:

1. Incomplete synthesis of H-2 products at the stage of development.
2. Portions of the H-2 molecules could be masked.
3. Synthesis or expression of H-2 antigens other than those specified by the $H\text{-}2^{q}$ or $H\text{-}2^{s}$ haplotypes could occur.

The last theory was based upon earlier ideas that H-2 antigens which can vary epigenetically are involved in specifying position during development (Edelman 1976). In this view, quantitative variations between different lines derived from four day old mouse blastocysts represent differences in the modified H-2 antigens expressed by the cell lines. More experimental evidence will be required to substantiate these ideas which remain only speculative at present.

Whatever the underlying mechanisms may be, it is apparent that changes in adhesiveness do occur during differentiation from the work of Bellairs (see Bellairs et al, 1978). This showed that cells from ectoderm, neural plate and somites become more adhesive with differentiation. Cells from the segmented mesoderm are more adhesive to substrates and to other cells, than those from the unsegmented mesoderm. Cells from the ectoderm are more adhesive to each other and to substrates than neural cells. These results support the idea that an increase in cell-cell adhesiveness of the mesoderm plays a role in the process of segmentation. There may even be regions of high and low adhesiveness which could be important in somite formation. In conclusion, it may be that the differential expression of H-2 products throughout development is responsible for changes in adhesivity but much remains to be done to substantiate this theory.

1.6. Experimental Format.

From the previous, H-2 is implicated in many roles involving cellular interactions and although this has been investigated using many different techniques, no clear answer has

emerged from their results. In the work presented in this thesis, I applied variations of standard methods to try to further elucidate the role of H-2 in cellular interactions. The variations used were as follows. Plastic surfaces were chemically modified following the methods of Aplin and Hughes (1981) in order to attach H-2 fragments to them. Experiments were carried out to investigate the variability in cell adhesion and spreading of cells to these syngeneic and allogeneic fragment - coated substrates. It has been previously shown that cells shed H-2 fragments into the medium (see for instance Emerson et al, 1980). Alternatively, they can be prepared from crude membrane preparations (Turner et al, 1975) and these can be purified by similar methods used by Parham et al (1979) for HL-A purification. After studying the interactions of cells with substrate bound H-2 fragments, the next logical step was to examine the effect of preincubation of cells with these fragments on their ability to adhere to plain glass substrates. These results are presented in the text. Both the number of cells adhering and their spread area was calculated since little work appears to have been done comparing allogeneic effects on cell spreading abilities. The limitations of these studies is observed in the fact that H-2 antigens may play an indirect role in adhesion and such techniques would not distinguish direct effects from indirect ones.

I decided to reinvestigate the problems raised in this discussion by mixing cells of the same tissue type but which differed in H-2 haplotype. Similar experiments have been reported by McClay and Gooding (1978) as previously discussed. Their

failure to obtain evidence for sorting out may be attributable to the limited number of loci examined in their system. Cell adhesive interactions have often been studied by the ability of unlike cells to form aggregates in gyratory cultures. These methods were first employed by Moscona and Moscona (1952), Moscona (1960) and Steinberg (1964), although aggregation diameter assays such as these present certain disadvantages for the quantitative study of strengths of adhesion (Curtis, 1973). Here however, quantitative results were obtained from aggregates formed from suspensions of two different histocompatibility types of cells obtained from murine lung. These cells possessed many endothelial-like qualities (Barnhart and Baechler, 1978) and one of the two H-2 types used were pre-labelled with tritiated thymidine that they may be envisaged in the sectioned aggregate. Finally, H-2 antigens were detected on the surface of cells using monoclonal antibodies in immunofluorescence techniques, or by complement-mediated cytotoxicity tests. These results were interesting in relation to current ideas on H-2 expression both on homozygous and heterozygous cell types (Eskinazi et al, 1981; Robertson, 1982; Nakano et al, 1981: O'Neill and Blanden, 1979; etc.). Furthermore, the demonstration of adsorption of allogeneic Iα molecules, in an antigenically recognisable form, on the surface of peritoneal exudate cells could have important implications in the evaluation of the present models of cell interaction in the immune response, and for studies on the mechanisms of antigenic presentation and graft rejection.

CHAPTER 2. MATERIALS AND METHODS.

MATERIALS AND METHODS

All chemicals used were of 'Analar' grade where possible. Media were sterilised by autoclaving and were stored at 4°C except those which contained serum, which were stored at -20°C. Penicillin / Streptomycin was obtained from Flow Labs.

Hepes Saline (HS).

NaCl	40g
KCl	2g
D(+) Glucose	5g
0.5% Phenol Red	10mls
*Hepes	11.92g

The above were made up to 5 litres with distilled water and the pH was adjusted to 7.5. (*Hepes is N - 2 - hydroxyethylpiperazine - N' - 2 - ethane sulphonic acid and was obtained from Cambridge Research Biochemicals).

Hanks-Hepes Saline.(HHS)

Hepes Saline	5 litres
$2.H_2O.CaCl_2$.	0.93g
$6.H_2O.MgCl_2$.	1.0g

The pH was adjusted to 7.5.

Hams F10 +ITS + 3% HIFCS.

Hams F10 x10 concentrate (Flow Labs).	10mls
L-Glutamine 200mM (Flow Labs).	1ml
Penicillin / Streptomycin 5,000 units / ml	2mls
Hepes Saline	90mls
*Insulin / Transferrin / Selenium	0.25mls.

Heat inactivated foetal calf serum 3mls

The above were mixed in sterile conditions and then stored at -20°C. Sterility checks were performed on these media, by incubating 10mls of the final solution at 37°C, for 48 hours, then observing the bottles for bacteriological or fungal contamination. (* See page 39)

Eagles Medium + F.C.S.

*Glasgow Modification of Eagles MEM. (Flow)	180mls
Heat- Inactivated F.C.S.	20mls
L-Glutamine	1ml
Penicillin / Streptomycin 5,000 units/ml	4mls
$Tryptose Phosphate Broth (Difco)	20mls

$Tryptose Phosphate Broth was obtained from the departmental stocks and was made by dissolving 29.5g in 1 litre of distilled water. *Glasgow Modified Eagles medium is given in Stoker and MacPherson (1964).

Versene. (EDTA-Saline).

NaCl	40g
KCl	1g
Disodium Hydrogen Orthophosphate	5.75g
Potassium Dihydrogen Orthophosphate	1g
Phenol Red 0.5%	15ml
E.D.T.A.	1g

The above were made up to 5 litres with distilled water and the pH was adjusted to 7.4.

Trypsin

0.25% Trypsin (Difco, 1:250) was made up in Tris saline. This solution was obtained from the Virology Department, University of Glasgow. For use , it was diluted 1 in 5 in Versene.

Calcium And Magnesium Free Salt Solution. (CMF).

NaCl	70g
KCl	3.7g
$Na_2HPO_4.12H_2O$	3.0g
KH_2PO_4	2.4g
D(+) Glucose	10g
*TRIS	30g
Phenol Red (0.5%)	40mls

The above were made up to 1 litre with distilled water and the pH was adjusted to 7.85. Before use, it was diluted 1 in 10 with sterile distilled water. (*TRIS is hydroxymethyl amino methane).

Ammonium Chloride.

This solution was prepared in saline and used according to the method given in Mishell and Shiigi (1980).

Trypan Blue.

This was prepared using the method given in Mishell and Shiigi (1980). Trypan Blue was obtained from BDH.

Dulbecco's Phosphate Buffered Saline (PBS).

Solution A.

NaCl	8g
KCl	0.2g

Na_2HPO_4	1.15g
KH_2PO_4	0.2g

The above were made up to 800mls with distilled water. The pH was adjusted to 7.4 using 1 or 2 drops of 5M NaOH.

Solution B.

100mls of 0.1% $CaCl_2.2H_2O$

Solution C.

100mls of 0.1% $MgCl_2.6H_2O$

Working Solution.

800mls of A were asceptically mixed with 100mls each of B and C. (N.B. for Calcium and Magnesium free, solution A alone was used).

Fluorescein Diacetate (F.D.A.).

Solutions were made and used as given in Mishell and Shiigi (1980).

I.T.S. Medium (Insulin / Transferrin / Selenium)..

250 mg bovine Insulin (Sigma) was dissolved in 10mls of 0.01M Sodium Acetate buffer (pH 3), then run through a calibrated 30ml P6 Biogel (Biorad) column using the same buffer. The concentration of Insulin was estimated then chelated with EDTA to remove ionic zinc and other heavy metals. The mixture was dialyzed overnight against acetate buffer to remove the EDTA which had bound Zinc contaminants, and the amount of Insulin remaining was estimated again. The same amount of human Transferrin (Sigma) was added, and saturated by adding a stochiometric quantity of ferric Nitrilotriacetate. From the known concentration of Insulin /

Transferrin in this mixture, one thousandth the concentration of Selenium was added in the form of Sodium Selenite. This was then set to an Insulin concentration of 0.5mg / ml and 0.5mls was added to 200mls Of medium.

Protein Estimations - Reagents.

See Bradford (1976) for details of methods.

Siliconised Flasks.

10ml conical flasks were cleaned and dried then rinsed three times with 1% silicone DC1107 at a 1% concentration in ethyl acetate and dried in a stream of Nitrogen. They were then baked in an oven at 150°C for 2 hours and oven sterilised before use.

Formol Saline.

40% Formaldehyde	100mls
NaH_2PO_4 (anhydrous)	6.5g
Na_2HPO_4 (anhydrous)	4.0g
Distilled Water	900mls
Mg_2CO_3	(excess)

The above were mixed together and the precipitate was allowed to settle before use.

Monoclonal Antibodies.

The appropriate cell lines were obtained from the American Type Tissue Collection (Maryland, USA), and also as gifts from Dr. B. Askonas, Dept of Immunology, NIMR, Mill Hill, London. The following clones were used;

11.4.1.

This antibody is directed against the K^k allele and was

originally separated by Oi et al, (1978). It was used at a dilution of 1 in 1000 in PBS.

15.5.5.

This monoclonal reacts with the D^k allele. For further information, see Ozato (1980). Used at 1 in 1000 in PBS.

141.34.

This also reacts with the D^k allele and was used at 1 in 1000 in PBS.

Cell cultures were maintained in Hams F10 + 3% HIFCS + ITS. Culture supernatants were collected and stored at -20°C until at least 1 litre had been obtained. Antibodies were recovered by the method outlined in Mishell and Shiigi (1980).

Staining Procedures.

Preparations to be stained were rehydrated and were stained by one of the following methods:

1.Kenacid Stain.

The receipe for this stain is given elsewhere (see for instance Laemmli, 1970).Coomassie Brilliant Blue R250 (0.1%) was obtained from BDH. 1ml was added to the preparation and left for 10 minutes. It was then washed off with water and preparations were allowed to dry in air. They were then mounted using Clearmount.

2.Iron-Alum Haematoxylin.

3.Masson's Trichrome Stain.

Details of these stains and procedures are given in Silverton and Anderson (1961) and the fifth edition of Biological Stains and Staining Methods (1972). After staining, they were

dehydrated in alcohol (or left to dry if the stain was alcohol soluble) and mounted in Clearmount.

Standard Histological Techniques.

Tissues were fixed in Formol Saline overnight. These were then dehydrated through a graded series of alcohols and cleared in two changes of xylene then placed in paraffin wax at 56°C. After four changes of wax, they were finally embedded in the same, and sectioned on a Jung Rotary Microtome in the Histology Department of Glasgow Royal Infirmary. Sections of 5µm were obtained which were rehydrated and stained as required.

Animals

Animals were obtained from OLAC, animal suppliers (Bicester) and were maintained in the Departmental stocks in the Animal House, Dept of Neurology, Southern General Hospital, Glasgow. Within an experiment, all animals used were of the same sex, since it was necessary to exclude any effects on the results due to H-Y antigen interactions. Immediately prior to use, the animals were killed by neck dislocation unless peritoneal cells were required. In this case, animals were lethally etherised. This resulted in samples of peritoneal exudate cells which were not contaminated with blood.

All experiments were conducted within the restrictions of the 1876 Cruelty To Animals Act, covered by the licence to experiment on living animals and certificates A and B.

Haplotypes of congenic strains are those quoted by Klein et al (1978).

Fluorescence MIcroscope.

Fluorescence microscopy was done using a 50x oil immersion objective on a Vickers M41 microscope fitted with epi-illumination from a HBO 200W mercury arc lamp and appropriate excitation and barrier filters for the fluorochrome used.

Polyacrylamide Gel Electrophoresis (SDS.PAGE).

SDS-PAGE reagents and techniques used were as described by Laemmli (1970). All reagents were electrophoretically pure and were obtained from the following sources: acrylamide and bis-acrylamide from Koch-Light; sodium dodecyl sulphate (SDS) and Coomassie Brilliant Blue R (C.I.42660) from BDH; ammonium persulphate, bromophenol blue, and N,N,N',N',-tetramethylethylenediamine (TEMED) from Bio-Rad; Tris (hydroxymethyl) aminomethane (tris) and glycine from Sigma.

Silver Staining Of Gels.

Details of the reagents and procedure were followed as given in Oakley et al (1980).

Electron Microscopy.

Endothelial cell monolayers were fixed in situ in 4% glutaraldehyde in Sorensen's Buffer at pH 7.35, for several hours prior to secondary fixation in 1% Osmium Tetroxide. The coverslips were then subjected to dehydration through alcohol to propylene oxide. Several changes of embedding resin were applied and they were then inverted onto a gelatin capsule filled to the top with embedding medium thus making three layers, ie.coverslip, cells, and capsule. The preparation was then polymerised at 70°C

overnight. Embedding resin was Emix (Emscope Ltd, Kent, England). The capsule and its coverslip top were placed in a flask of liquid nitrogen, and the coverslip immediately detached itself leaving the cell monolayer firmly embedded in the top of the polymerised capsule. The block was then mounted in an LKB ultramicrotome IV and ultra - thin sections (50nm) were obtained using a diamond knife. The sections were mounted on copper grids and stained conventionally with orange acetate and lead citrate. They were examined in a Phillips EM 200 transmission electron microscope and recorded on Kodak electron image film using an accelerating voltage of 60KV. Printing was achieved using a Besler enlarger in conjunction with the Ilfospeed printing system. Standard EM techniques are outlined in Hayat, 1972.

General Tissue Culture Methods.

All manipulations in tissue culture were carried out under sterile conditions, in a laminar flow hood. Foetal calf serums were heat inactivated (HIFCS) at 56°C for 1 hour in a water bath to destroy complement activity and sterilised by passage through a millepore filter (Millepore Corporation, USA) of pore size 0.22µm. Glassware was washed in decon and distilled water then sterilised by heating at 160°C for 2 hours. Instruments were sterilised in 70% ethanol then flamed. Millipore filters and univerals were autoclaved. All autoclaving was carried out at 15lbs / sq. in. for 15 mins.

Cultures were maintained in 25 cm^2 tissue culture flasks (Sterilin or Nunclon). Wherever possible, low serum medium (2.5%) was used as it has been shown that serum affects the adhesiveness of cell types differentially (Curtis, 1965). This could be of particular importance when cell suspensions contained mixed cell types and the ability of these to adhere to substrata was being observed.

Preparation of Lung Explants.

Lungs were obtained from two mice under sterile conditions and were cut into fragments approx 1mm^2 in Hanks-Hepes. After two washes with CMF, they were transferred by pasteur pipette to a conical flask containing trypsin/versene, and were mixed at 37°C for approximately one hour, until the mixture appeared opaque. It was then transferred to a sterile universal, washed twice in Hams F10 + 10% HIFCS and resuspended in 5mls of

fresh medium containing 20% HIFCS. The suspension was transferred into two 25ml culture flasks and incubated at 37°C for 24 hours to allow the cells to adhere. Unattached cells and tissue fragments were carefully removed with a pasteur pipette and 10mls of fresh medium containing 3% serum + ITS were added.

When primary cultures reached confluence, they were washed twice with CMF then with trypsin/versene. This was poured off, and cultures were examined at 37°C over the next few minutes. Fibroblasts tended to detach from the substrate first, and approximately 70% pure cultures of these could be obtained by stopping the trypsinisation after two minutes, transferring the suspended cells to another flask. The next cell type to detach from the flasks with trypsin treatment were endothelial-like cells. These detached after 2-7 minutes. Some cells seemed to be fairly trypsin - resistant and remained on the flask after 20 minutes trypsinisation. These probably included the macrophages, and were usually discarded. Endothelial - like cells and fibroblasts were cultured separately and purified by repeating the trypsinisation procedure discussed above. Trypsinisation was stopped in all cases by adding HIFCS dropwise then washing twice in Hams F10 with 10% HIFCS. The cultures were resuspended in Hams F10 + ITS + 3% HIFCS and were maintained at 37°C until required. Fibroblast and endothelial - like cultures were obtained which were more than 90% pure on the basis of morphological criteria.

Preparation Of Coverslip Cultures.

Cell suspensions were prepared by trypsinisation with 1 : 4 trypsin / versene and were diluted in Hams F10 + 10% HIFCS at

a concentration of 3×10^4 cells per ml. 1ml was then added to a well containing a sterile glass coverslip and the containers sealed and incubated at 37°C as required.

Categorisation Of Endothelial - Like Fraction.

The endothelial - like cells were examined under transmission electron microscopy by Mr. C. Mucci (Department of Cell Biology) and by Dr. J. Anderson and Dr. P. Toner (E. M. Unit of Glasgow Royal Infirmary) to determine whether the internal organisation was typical of endothelial cells, fibroblasts or epithelial cells. To determine whether the endothelial - like cells were in fact of epithelial lineage, an examination of the intermediate filament proteins present in these cells was made by Dr J. G. Edwards and Mr. A. Hart (Department of Cell Biology) using SDS- PAGE techniques. Endothelial and fibroblastic cells have vimentin intermediate filament proteins whereas epithelial cells have cytokeratins (Franke et al, 1979). The morphological appearance of the cells were compared to pig aortic endothelium since endothelial cells tend to have a typical pavement - like morphology (Barnhart et al, 1978). Since endothelial cells are known to contain Factor VIII antigen and angiotensin - converting enzyme (Barnhart et al 1978) the cells were examined for the presence of these proteins using immunofluorescence and spectrophotometry techniques respectively. Professor A.S.G. Curtis performed the work on angiotensin converting enzyme according to the method of Cushman and Cheung (1971).

Preparation Of Pig Aortic Endothelium.

Pig aortas were obtained freshly from the animals

immediately after killing in a local abertoire. A cannula was inserted in one end and tied in place. The other end was sealed completely and the aorta filled with HHS. In a sterile environment the intercostal arteries were sealed with crocodile clips and the aorta was washed in HHS. It was then filled with 0.5mg/ml collagenase (Sigma) and left for 20 minutes at 37°C. After carefully rinsing in Hanks Hepes the aorta was filled with Eagles medium + 10% HIFCS and was rubbed gently between finger and thumb to dislodge the endothelial cell lining. This solution was then transferred to sterile culture flasks, gased in a 5% CO_2 atmosphere and cultured at 37°C. The medium was changed after 24 hours and cells were allowed to grow to confluence (3 - 7 days). These were then used for comparison to the endothelial - like cells separated from murine lung cultures.

Cellular Microfilament Preparation For SDS-PAGE.

Intermediate filament preparations were made according to the method given in Brown et al (1976). High salt treatment for removal of actin, histones and DNA was applied according to previously published methods (see for instance Franke et al, 1979b). Phenyl - Methyl Sulphonyl Fluoride and Tosyl - Argenine Methyl Ester were obtained from Sigma. 200µl of samples were diluted with boiling mix and were added to the SDS gels.

Immunofluorescent Staining Of Factor VIII Antigen.

Confluent coverslip cultures of lung endothelial - like cells were fixed for 30 seconds in cold Methanol (at -20°C) and washed in PBS. Rabbit anti - Human Factor VIII (Nordic Immunologicals) diluted with PBS was added to the cells and was

incubated at 4°C for two hours. This was then washed off using four changes of PBS, and Sheep anti - rabbit (Nordic Immunological) Ig was added at a dilution of 1 in 40 in PBS. This second antibody was conjugated to FITC and after a further hour at 4°C, excess was washed off to prevent high background staining. After four washes in PBS, coverslips were placed on glass slides and a second clean coverslip was added. These preparations were examined under green excitation on the fluorescence microscope. Only those cells showing perinuclear staining were counted as positive. Results were expressed as % labelling of the total.

Labelling Of H-2 Antigens Using Monoclonal Antibodies.

Subconfluent coverslip cultures of endothelial - like cells were obtained and washed in PBS. They were then placed in 1.5cm wells containing 1ml of the 1 in 1000 dilution of 11.4.1 anti K^k and placed at 4°C for two hours. It was unlikely that the cells could ingest antibody by phagocytosis at this temperature and consequently any antibody detected on the cell surface was likely to be bound to K^k products.

Immunofluorescent Double Staining Techniques.

Coverslip cultures were incubated with monoclonal antibody as above, and were washed three times in PBS to remove unbound antibody. The were then incubated with 0.5mls Rabbit anti - mouse Fab' (Nordic Immunologicals) bound to Fluorescein Isothiocyanate (FITC) at a dilution of 1 in 40 in PBS for one hour at 4°C. After four washes in PBS, the coverslips were examined using green excitation under the fluorescence microscope.

Complement Fixation Tests.

These tests were carried out on the monoclonal antibodies, purified as above, to check that they could bind complement for use in complement - mediated cytotoxicity studies. The technique used was as follows.

Sensitisation Of Sheep Red Blood Cells.

0.1mls of washed 4% sheep red blood cells (SRBC) were added to various dilutions (1/125 - 1/8000) of rabbit anti - sheep haemolysin (Flow Labs) and incubated at 37°C for 15 minutes.

Preparation Of Endothelial-Like Cell-Monoclonal Antibody Complex.

Sub - confluent coverslip cultures of B10A cells were washed three times in Tris Saline, and were added to dishes containing a range of dilutions of the monoclonal antibody 11.4.1. (anti K^k). These were incubated overnight at 4°C, then 50µl of guinea pig complement was added to each. After incubating for 40 minutes at 37°C the culture supernatant was removed for use in the complement fixation test.

Haemolysin Assay.

0.1mls of sensitised SRBC was added to 0.1ml of 1/20 complement (50µl / ml) in a small round - bottomed well in a "World Health Organisation" (WHO) tray obtained from the Immunology Dept, Western Infirmary, Glasgow. After 30 minutes incubation at 37°C the wells were scored for haemolysis. Where no haemolysis (ie. negative result) had occured, a button of cells were seen.The results of this assay provided the correct dilution of haemolysin for use in the fixation test.

Complement Fixation Test.

Either a single optimal dilution of haemolysin (obtained from the above) assay was used, or a range around this dilution. 0.1mls of each dilution of sensitised SRBC and 0.1mls of the supernatant from the endothelial cell cultures containing monoclonal antibody and complement was added to a WHO tray. The mixtures were incubated at 37°C for 30 minutes with regular mixing, then each well was scored for % lysis.

Complement-Mediated Cytotoxicity.

Guinea Pig Complement (Wellcome) was reconstituted using sterile water. Preliminary experiments were performed to find the optimal dilutions of antibody and complement which gave the greatest % specific lysis of targets during the incubation time, and also to find the optimal incubation time. Both 11.4.1 and 15.5.5s antibodies were found to have an optimal dilution of 1 in 1000 with an optimal incubation time of 2 hours at 4°C, and 50µl of complement was used for 40 minutes at 37°C. Controls were included to check that neither antibody nor complement alone caused non-specific cytotoxicity. To check that contaminants were not present in the medium, a control of medium alone was also included and in experiments where more than 25% of the cells in this control were lysed, the experiment was discarded. Coverslip cultures were incubated with monoclonal antibodies as above and 50 µl of complement was added to each. They were placed at 37°C for 40 mins to allow lysis to occur then viable counts were performed using either the Trypan Blue exclusion or F.D.A. methods (Mishell and Shiigi 1980).

Preparation Of Peritoneal Exudate And Spleen Cells

Both cell types were prepared using the method given in Mishell and Shiigi (1980). Cells were resuspended in Hams F10 + I.T.S. + 3% HIFCS. Viabilities were checked using Trypan Blue exclusion methods and were usually about 95% viable. When a PEC preparation was heavily contaminated with blood, it was not used since although red cells can be removed by various methods, white blood cells would still be present which could affect results. Red blood cells were removed from spleen cell preparations using ammonium chloride as previously mentioned.

Preparation Of H-2 Fragments

Spleen cells were suspended at 5×10^7 / ml in serum - free RPMI and were allowed to incubate for 1-2 hours at 37°C. During this time the viability of the cells did not fall significantly. The supernatant obtained after centrifugation at 600-700g. was then filtered through a sterile 0.22µm Millepore filter and subsequently through an Amicon UM10 pressure dialysis membrane at 55 p.s.i. of Nitrogen. The resulting filtrate should not in theory contain any components with a molecular weight over 10,000 Daltons but on further examination the limit for these filters on H-2 fragments seemed to be about 40K Daltons (Curtis and Davies 1982). This fraction was then concentrated on a UM2 membrane (1,000 daltons pore size) to nearly a tenth of its original volume. The semi - pure fraction was then assayed for its ability to reduce the adhesiveness of the opposite cell type (i.e. if the fraction was purified from T cells it would reduce the adhesiveness of B cells of the same H-2 type - for details see

Curtis and Davies, 1982). Following a successful assay, the factor was further purified on a Biogel P-30 column equilibrated with 10mM Hepes water at pH 7.4. The resulting protein peak was taken and reassayed for de-adhesion activity and usually contained 150-200µg of protein per ml.The details of the adhesion and assay system are given in Curtis and De.Sousa (1975). The activity of the factors is expressed in units such that 10^4 units/ml depresses the adhesion of 10^6 B cells to zero. This activity unit refers to the adhesiveness measured in medium 199. Factors were stored at -20°C and were frozen-thawed no more than once before use. They have been previously shown to be non-toxic by the above-mentioned workers. Factors were affinity purified following the method given in Curtis and Davies (1981).

Attachment Of H-2 Fragments To Chemically-Modified Surfaces.

Glass coverslips were modified according to the method outlned by Aplin and Hughes (1981). H-2 fragments were prepared as outlined above and were allowed to attach to the coverslips for 1 hour. They were washed moderately well with PBS and kept at 4°C until required. A small amount of PBS was left on to keep them moist until required.

Detection Of H-2 Fragments On Chemically-Modified Surfaces.

To check that H-2 fragments had stuck to chemically - modified surfaces, the following experiment was done. Coverslips were prepared as usual, and H-2D fragments were added from B10 D^b) and Balb/k (D^k) strains. HIFCS and PBS - treated control were also included. Monoclonal anti -D^b (15.5.5 clone) and anti D^k (141.34 clone) were added to duplicate coverslips at dilutions of 1 in 1000 and were allowed to incubate for 2 hours at 20°C. They

were washed thoroughly in PBS, then Rabbit anti - mouse Fab' antibody conjugated to FITC (Nordic Immunologicals) was added at a dilution of 1 in 40 in PBS. This was incubated at 20°C for 1 hour and washed four times in PBS. Coverslips were then examined "blind" under the fluorescence microscope.

Preparation Of Conditioned Medium.

Spleen cells and peritoneal exudate cells were prepared as above, and 1×10^7 cells were transferred into sterile tissue culture flasks in Hams F10 + I.T.S. + 3% HIFCS. They were incubated at 37°C for the desired time interval (24hrs, 48hrs, 72hrs etc). Cell suspensions were transferred to a sterile universal and centrifuged at 2000 r.p.m. for 5 mins. Supernatants were retained and were passed through sterile millepore filters of pore size 0.22µm. Protein estimations were performed on these and all were diluted to contain 1mg /ml of protein using sterile PBS. The supernatants were then frozen at -20°C until required.

Incubation Of PEC's With Conditioned Medium; Adhesion Assays.

Peritoneal exudate cells (PEC's) were prepared as above in HHS then were mixed with an equal amount of conditioned medium so that approximately 1×10^5 cells were in a final volume of 2mls. Containers were sealed and were placed on an Emscope rotary mixer at 37°C for thirty minutes. Preparations were washed three times in Hanks-Hepes Saline and finally resuspended in 1ml Hanks-Hepes Saline as above. Coverslips were prepared previously as follows. Firstly they were washed in Nitric acid for 20 minutes then washed in distilled water and finally rinsed in absolute ethanol. They were heat-sterilized before use and allowed to cool

down to room temperature. Cells were allowed to adhere to coverslips for 30mins at 37°C. The supernatant was then gently removed and the cultures were fixed in formal saline and stained with Kenacid for 10 minutes. They were washed, allowed to dry in air, then mounted in Clearmount. The total number of cells per field and total cell area were counted on the Quantimet Image Analyzing computer. Controls were included using unconditioned medium which had been kept at 37°C for 2 days and adjusted to a protein concentration of 1mg/ ml.

Detection Of $I\alpha^k$ Fragments On PEC's.

Washed cells were mixed with conditioned medium using the same method as outlined in the adhesion assay above. They were washed three times then reacted with polyclonal anti $I\alpha^k$ (Cedarlane Labs Ltd. CL8701 A.TH anti A.TL) diluted to 1/4000 in Hanks. After 30 minutes at 37°C, they were washed twice and mixed with 1/100 guinea pig complement and left for a further 30 minutes at 37°C. The complement was washed off and the cells were scored for percentage lysis using the Trypan Blue exclusion method as previously outlined. At least 200 cells were counted for each preparation.

To examine the adherent population of PEC's for the presence of $I\alpha^k$ fragments after preincubation in conditioned medium, PEC suspensions were allowed to adhere to microtitre plates in Hams F10 + 2.5% HIFCS + ITS for 2 hours at 37°C. Cell concentrations ranging from 1×10^5 to 4×10^5 cells / well were used. Adherent cells were washed three times then polyclonal antibody was applied as above.

Preparation Of Cells For Aggregation Studies.

For each H-2 strain type to be used, a confluent culture of lung explant cells were obtained. This was washed twice with CMF and once with Trypsin/Versene. After 5 minutes most cells were in suspension. HIFCS was added dropwise to stop the trypsinisation then the cells were washed twice in HHS. The cell suspension was then divided into two equal fractions. One was resuspended in Hams F10 + ITS + 3% HIFCS. The other was suspended in Eagles + ITS + 3% HIFCS + 5μCi/ml of Tritiated Thymidine (Radiochemical Centre, Amersham) at a cell concentration of approximately 5×10^6 per ml. (Tritiated Thymidine was supplied as [6-^{3}H] Thymidine in aqueous solution (TRA 61),). Both cultures were incubated at 37°C for 24 hrs, then the Tritiated Thymidine labelled culture was given a "cold chase" by resuspending the cells in three changes of Hams F10 + ITS + 3% HIFCS at 4°C with an hour between changes. This medium contains unlabelled Thymidine. Both cultures were trypsinised and cell suspensions were washed at least 3 times to remove any trace of Trypsin, using Hanks-Hepes Saline. They were resuspended in Hams F10 + ITS + 3% HIFCS at a concentration of 1×10^5 viable cells per ml and kept on ice.

Radioactive Counting

Endothelial cells were prepared as above. A suspension was prepared by trypsinisation and the cells were washed three times in Hanks Hepes Saline. 0.5mls of 0.3M NaOH was added to the final pellet and the cells were left overnight. This solution was then transferred to a plastic vial containing 10mls of Aquasol (New England Nuclear) as a scintillant. Vials were left to stand

overnight to allow the chemiluminescence resulting from this preparation technique to fall to a background level, then the samples were counted in a scintillation counter.

Beta Emission

This was measured in a Packard Tri-Carb 300 scintillation counter with a Tritium Efficiency of around 75% as determined from the SCR. Counting was for up to 5 minutes and background levels were automaticlly removed from the final counts received.

Aggregation Of Cells in Gyratory Cultures.

Between 3 and 5 x 10^5 cells were added to siliconised 10ml conical flasks in Hams F10 + I.T.S. + 3% H.I.F.C.S. The flasks were sealed by coating the stoppers with vaseline then were placed in a gyratory water bath at 37°C at a speed of 90 r.p.m. for 3 hrs. The speed was then reduced to 75 r.p.m. and the cells were left for 28 hours to allow aggregation to take place. The medium was then removed and aggregates were fixed in formal saline. Standard histological processing followed, and serial wax sections were prepared of all aggregates and were mounted on glass slides. The wax was removed by immersion in xylene. The surfaces were made more adhesive for the autoradiographic emulsion by dipping them in a filtered solution containing 5gm gelatin and 0.5gm chrome alum dissolved in 1 litre of distilled water. They were allowed to dry in dust free conditions and were processed for autoradiography.

Autoradiography.

Methods used are given in Rogers (1969). Emulsion (Ilford Nuclear Research) was made up prior to use with double distilled water (1 part emulsion : 2 parts water) and warmed to 56°C before use. Working by safe light in the dark room, slides were dipped in this solution and were leaned near vertical over foil until the emulsion had dried. The slides were transferred to plastic slide boxes which were then wrapped in foil and these were stored in a tin containing a dessicant at 4°C for 21-28 days to allow exposure to occur.

Slides were developed in Ilford phenisol diluted 1 in 4 with double distilled water at 20°C for 5-6 minutes. They were washed in double distilled water and fixed in 30% Amphix for 5 mins. They were then left to gently wash in running water for 30 minutes. Excess emulsion was wiped from the back of the slide and they were dried at room temperature. They were stained for 4 minutes using Iron - Alum Haematoxylin and differentiated in acid alcohol. They were then stained in Ponceau's stain for 3 minutes to enhance the nuclear staining, and differentiated in 1% Phosphomolybdic Acid. Slides were dehydrated in alcohol and cleared in xylene then mounted in clearmount. They were then examined under bright field optics using oil emmersion.

Counting Of Aggregate Sections

The percentage of labelled cells in the aggregates was obtained by counting approximately 200 cells and noting the number showing labelled nuclei. When comparing the number of cells labelled at the periphery with those labelled inside the

aggregate, it was important to define the peripheral layer as that layer of cells which were on the outside of the aggregate having only one surface in contact with other cells. Sometimes due to the plane of sectioning, or due to tearing of the sections, the perimeter of the section did not coincide with this outer layer. However, the true outer layer of cells appeared more flattened, often stained slightly darker than the internal cells, and were continuous, so could be easily distinguished from the torn edges.

Statistical Analysis.

Results were analysed as indicated using the standard t test for significance as given in Snedecor and Cochran (1967).

CHAPTER 3. CATEGORISATION OF ENDOTHELIAL - LIKE CELLS PURIFIED FROM MURINE LUNG CULTURES.

3.1. Categorisation Of Endothelial-Like Cells Purified From Murine Lung Cultures.

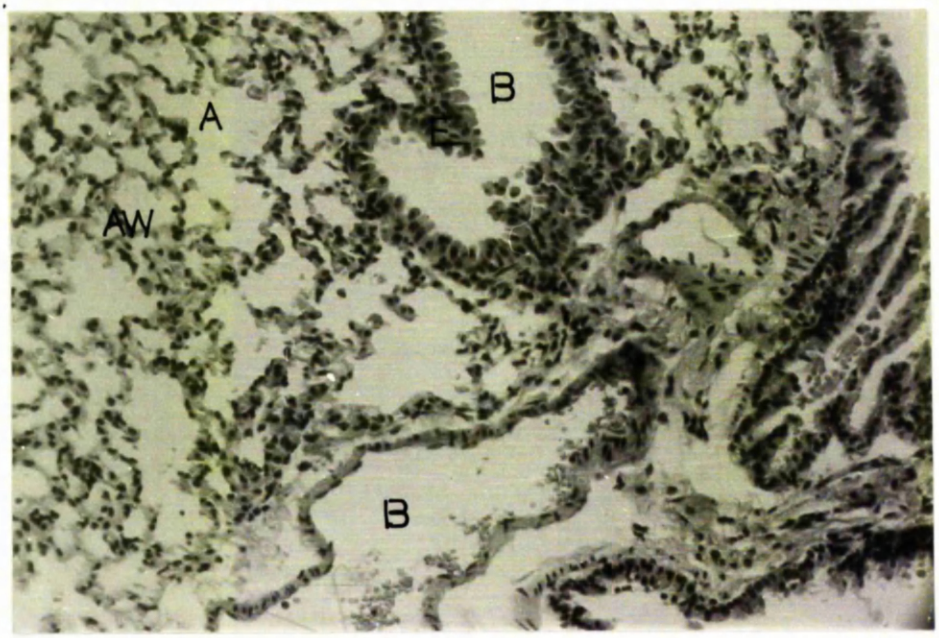
Lung tissue contains many different cell types (Plate 1) which differ in their susceptibility to trypsinisation in culture. After 10 minutes trypsinisation, endothelial cells and fibroblasts can be removed from primary cultures in suspension, whereas macrophages and epithelial cells remain attached to the substrate for a much longer time. Fibroblasts are in fact the most susceptible to such treatment and most can be removed from a mixed culture of cell types after 3 minutes treatment. This criterion was used to obtain cultures of endothelial cells of at least 90% purity on the basis of morphological appearance (Plate 2). The cultures were compared to pig aortic endothelium, and a typical 'cobblestone' appearance was seen in both (Plates 3 and 4).

Endothelial cells release angiotensin converting enzyme and contain perinuclear Factor VIII antigen (Barnhart et al, 1978). The first of these was tested for by Curtis (unpublished observations), and the endothelial - like cells were shown to secrete high levels of enzyme. The second protein was detected using Rabbit anti Human Factor VIII (R.a.HU.F.VIII) and Sheep anti Rabbit immunoglobulin conjugated to FITC (SAR-FITC) using an indirect immunofluorescence technique. The results of four experiments are given in Table 1.

Plate 1 (A and B).

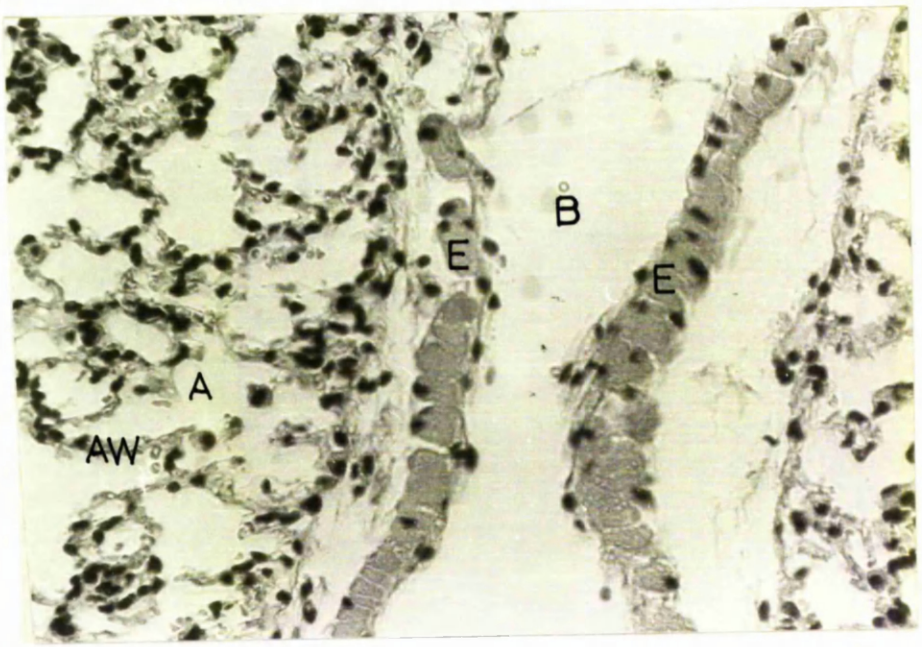
Haematoxylin and Eosin - stained sections of normal murine lung tissue, sectioned at 5µm showing general structure. Alveoli (A); alveolar wall (AW) comprising epithelial cells, phagocytes and endothelial cells; bronchiole (B) containing epithelial cells (E) and smooth muscle cells.

A.



1mm

B.



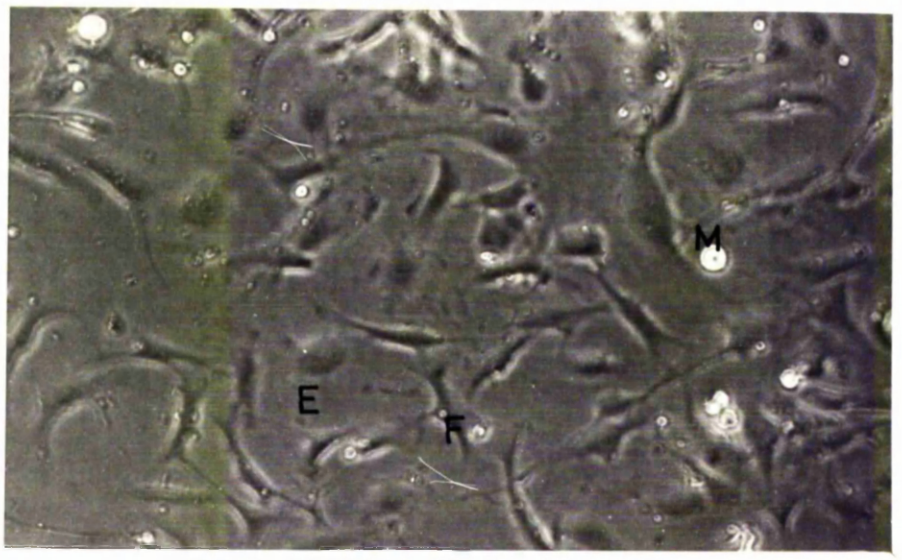
1mm

Plate 2.

A. Mixed culture of lung cells before selective trypsinisation, containing macrophages (M), endothelial - like cells (E) and fibroblasts (F).

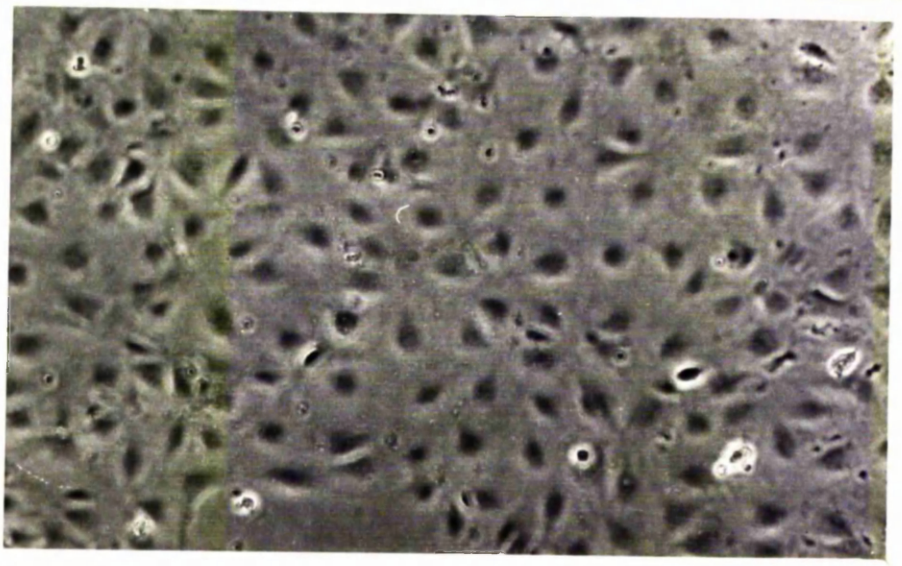
B. Same culture as in A above, but after selection by trypsinisation for the endothelial - like fraction.

A.



200 µm

B.



200 µm

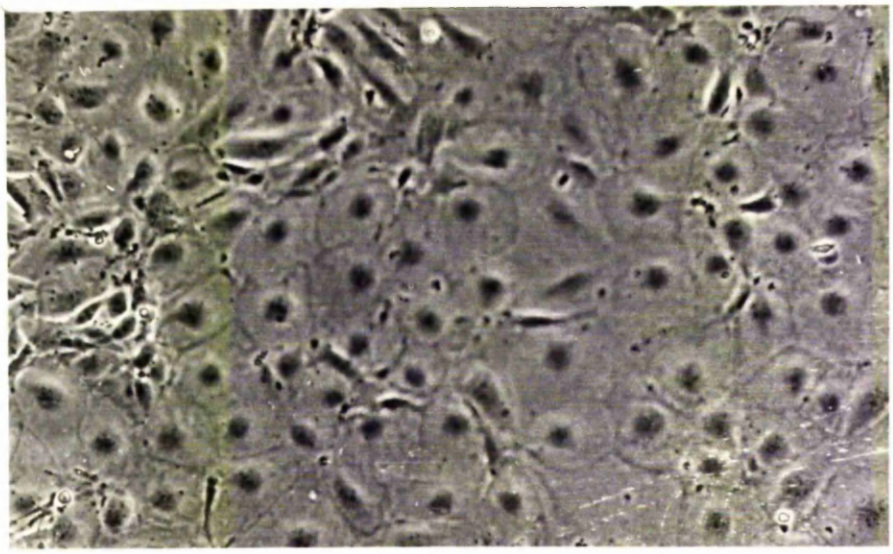
Plate 3.

Murine lung endothelial - like cells growing on tissue culture flasks, for comparison to pig aortic endothelium cultures (Plate 4). Phase contrast optics.

A. Low power view of cells showing typical pavement - like appearance.

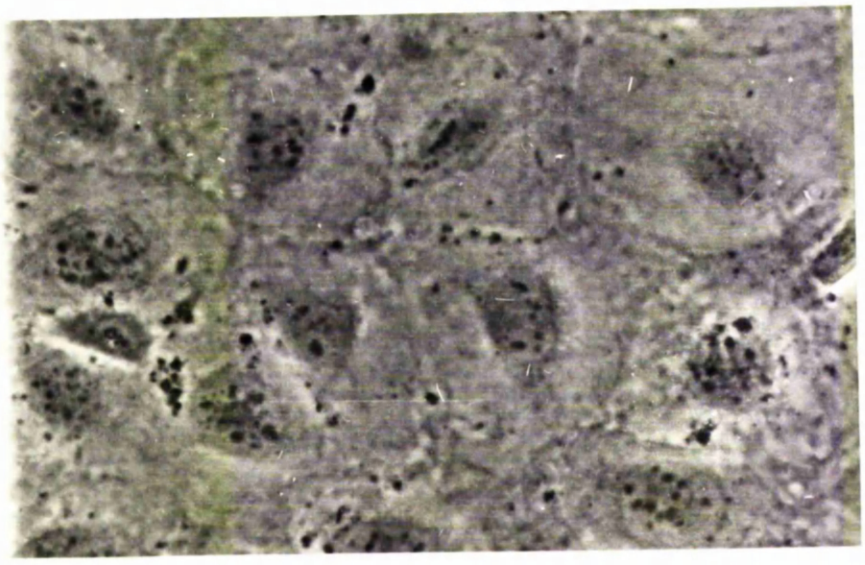
B. Higher power of cells to show large cytoplasmic area and granular nuclei.

A.



200μm

B.



200μm

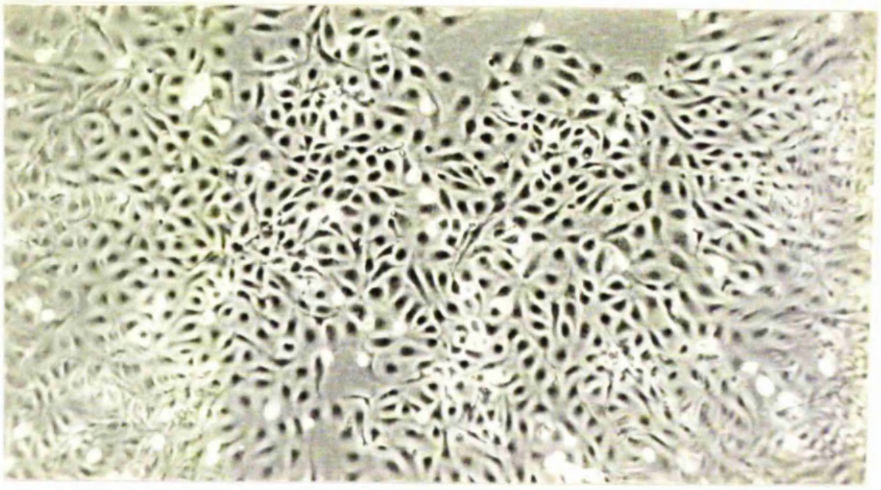
Plate 4.

Pig aortic endothelial cells growing on tissue culture flasks for comparison with murine endothelial - like cells. Phase contrast optics.

A - Low power view of cells demonstrating the 'cobblestone' appearance which is typical of endothelial cell cultures.

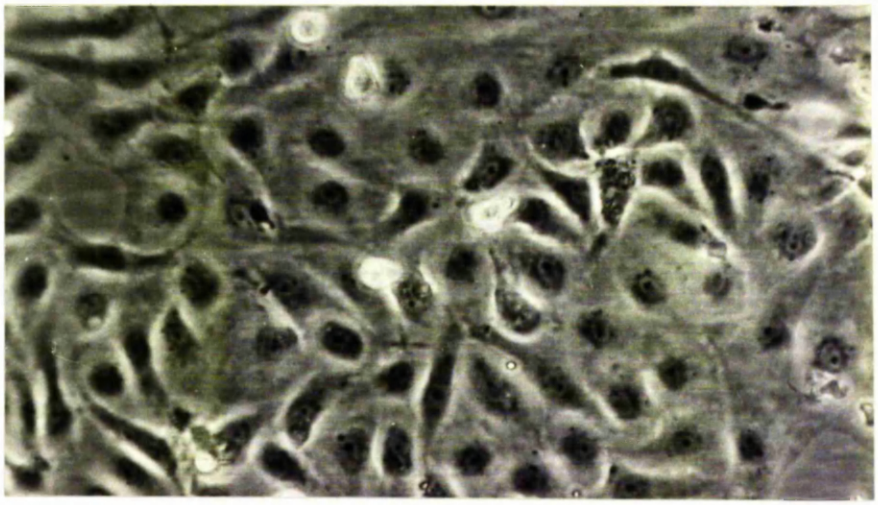
B - Higher power to show large cytoplasmic area with granular nuclei.

A.



1mm

B.



200μm

Table 1.

Presence of factor VIII antigen in endothelial - like cells purified from murine lung cultures.

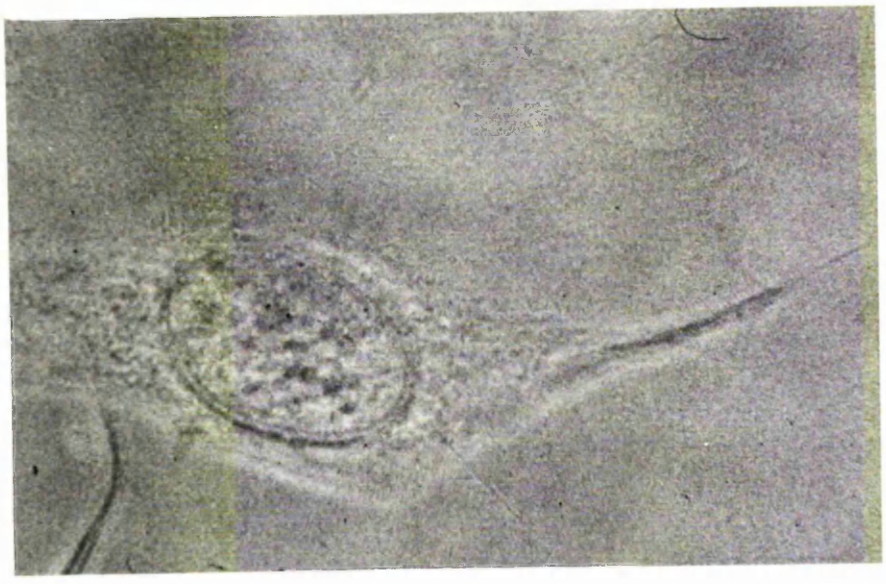
	Cell Type	
	Mouse	Mouse and
Antibody	Endo. Like.	BHK Fibroblasts.
RaHu FVIII 1/100	+	-
1/50	+	-
1/25	+	-
1/12.5	+	-
N.R. Serum 1/100	-	-
1/50	-	-
1/25	-	-
1/12.5	-	-
Phos. Buf. Saline	-	-
Tris Saline	-	-

RaHu FVIII = rabbit anti human Factor VIII. See text for interpretation of results. + indicates perinuclear fluorescence. N. R. Serum = normal rabbit serum. These results were read 'blind' to avoid bias and were conducted in conjunction with Mr. R. Renshaw of the same Department.

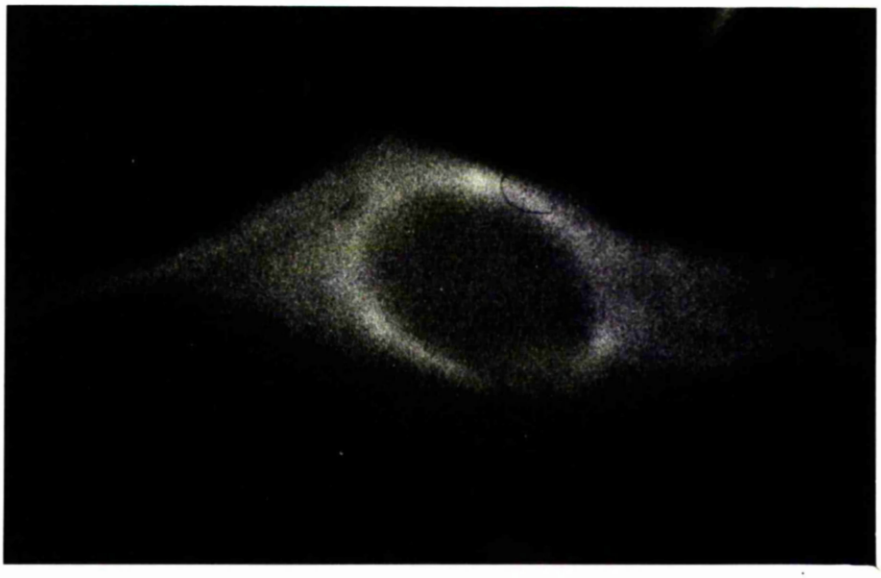
Plates 5 and 6.

Demonstrating the perinuclear staining of endothelial - like cells with rabbit anti Human Factor VIII, and sheep anti rabbit Ig conjugated with Fluorescein Isothiocyanate. A = phase contrast optics, and B = green excitation under the fluorescent microscope. Negative controls failed to fluoresce. These included murine lung and BHK 21 fibroblasts.

A.

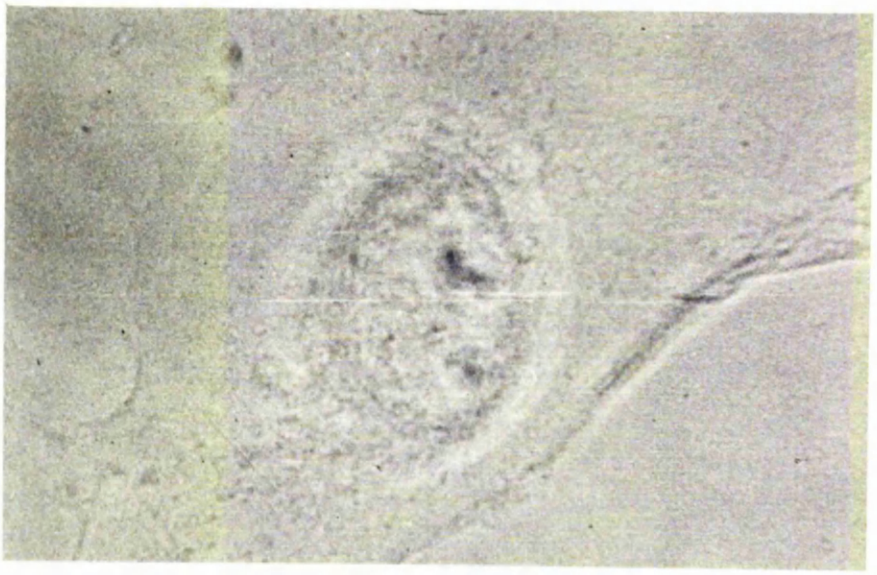


B.

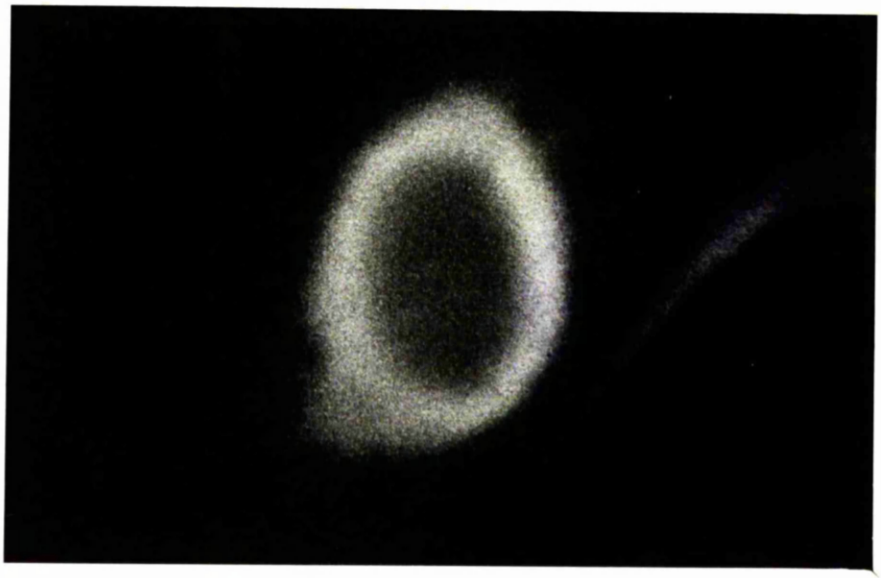


10 µm

A.



B.

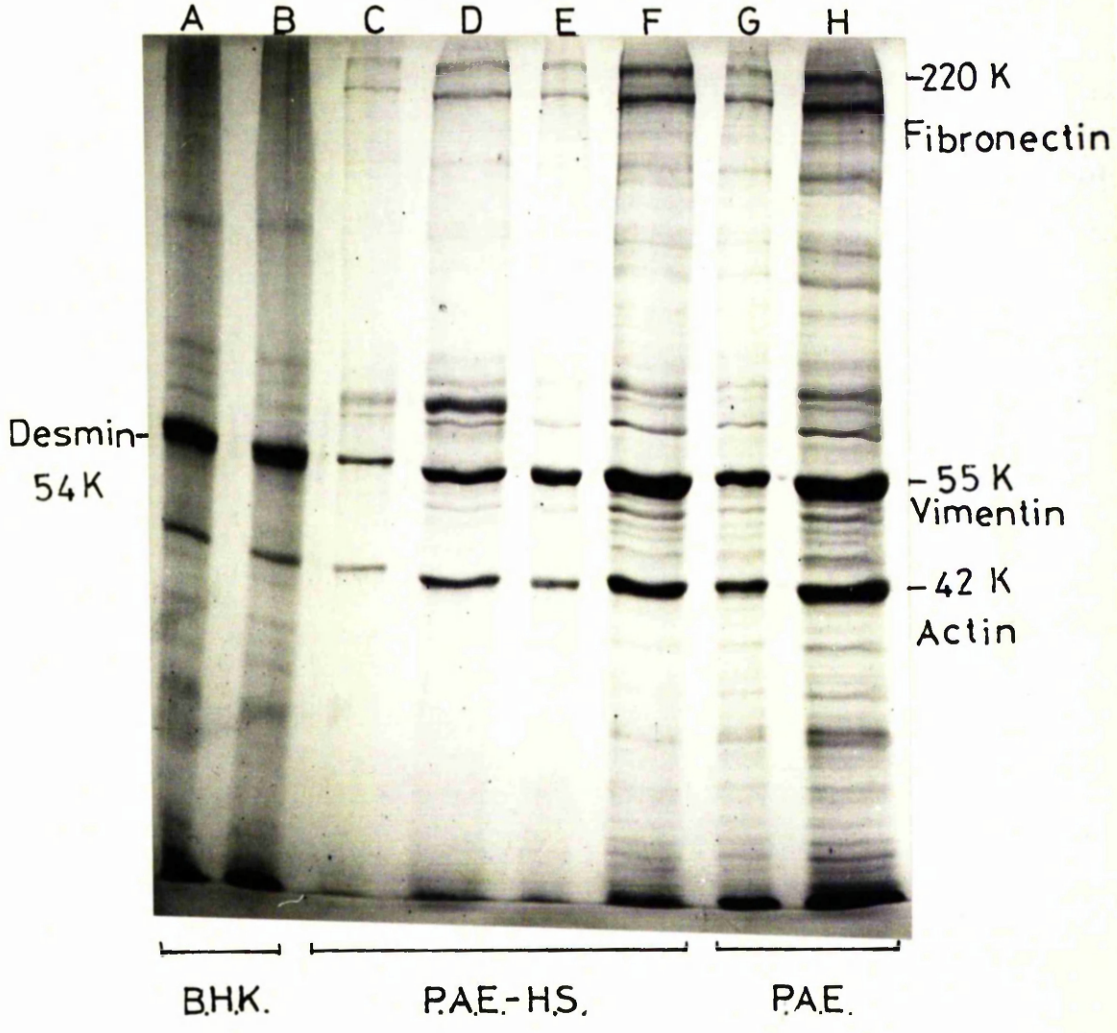


10 µm

Plate 7.

SDS - polyacrylamide gel electrophoresis of cytoskeleton preparations from BHK fibroblasts and pig aortic endothelial cells (P.A.E.). Murine lung endothelia showed analogous bands to those seen in the P.A.E. cultures. Tracks A and B = BHK 21 cells. Tracks C and D = High salt extraction of P.A.E. growing on a tissue culture flask. Tracks E and F = the same cells grown in plastic dishes, extracted with high salt. Tracks G and H show the same as E and F but not extracted with high salt. This high salt (ie. high ionic strength saline wash) treatment removes some of the cell debris and provides a partially purified preparation of intermediate filament proteins. 10µl and 40µl of pig aortic endothelium and BHK 21 preparations were added to consecutive lanes on the gel.

Bands of vimentin are seen at 55K and actin at 42K Daltons in all the preparations. Desmin bands are also seen in the BHK preparation. These run slightly below the vimentin at 54K Daltons.


A
B
C
D
E
F
G
H
-220 K
Fibronectin
Desmin-
54K
-55 K
Vimentin
-42 K
Actin
B.H.K.
P.A.E.-H.S.
P.A.E.

Before use, the R.a.HU.F.VIII and SAR-FITC were absorbed on mouse and BHK fibroblasts obtained from murine lung, since non-specific fluorescence was occaisionally seen with some antibody batches. The positive results given in Table 1 indicate that perinuclear fluorescence was seen. Examples of this are provided in Plates 5 and 6. Negative controls appeared black under the fluorescence microscopy, reflecting a lack of detectable Factor VIII antigen. Photographs of these have not been included in the text.

To distinguish the endothelial - like cells from epithelial cells, a comparison was made of the intermediate filament proteins of these cells with those of pig aortic endothelium and BHK fibroblasts. It is held that cells of an epithelial lineage have cytokeratin intermediate filament proteins, whereas endothelial and fibroblastic cells have predominantly vimentin intermediate filament proteins. SDS-PAGE methods were used, and the gels were subsequently stained with Kenacid Blue. Both pig aortic endothelium and murine endothelial - like cells showed vimentin intermediate filament proteins as compared to BHK fibroblasts. No cytokeratins were detected in any of these lines, which correlates with the failure to detect desmosomes in EM sections of the cells. Plate 7 shows an example of these SDS-PAGE results, in which pig aotic endothelium is compared to BHK cells in terms of intermediate filament proteins.

Transmission electron microscopy of coverslip cultures of the endothelial - like fraction was performed as previously stated by Dr. J. Anderson and interpreted by Dr. P. Toner of the EM Unit, Pathology Dept. Glasgow Royal Infirmary. The sections

showed (Plates 8, 9, 10, 11, 12) that the internal organisation of rough endoplasmic reticulum and ribosomes were consistant with an endothelial phenotype. Many free ribosomes were seen in the cytoplasm, but there was no significant amount of rough endoplastic reticulum unlike that which is often seen in fibroblastic cells. Endocytotic coated vesicles were present which are also found in macrophages, but the general layout of the organelles was not typical of either macrophages nor fibroblasts. Many microfilaments and microtubles were seen in the cells, including intermediate filaments. As SDS-PAGE and transmission electron microscopy was carried out on cultures of the same lineage, results can be correlated directly i.e; the predominant intermediate filament proteins were identified electrophoretically as Vimentin. The surface area of the cells was irregular suggesting a functional lability. Many pseudopodia were present, usually seen in cross-section. These characteristics indicate that the cells may be of an endothelial lineage.

Transmission electron micrscopy work conducted by Mr. C. Mucci in the Department of Cell Biology failed to find desmosomes as previously stated. Some cellular inclusions were seen which were comparable to Weibel-Palade bodies in terms of appearance and size (Plate 8b).

Plate 8.

Transmission electron micrograph of endothelial - like cells purified from murine lung cultures. M = mitochondria; M Fil. = microfilaments; PV = phagocytotic (endocytotic) vesicle; CP = coated pits, which are involved in endocytosis. Magnification 23,600 x. Scale bar = 1µm.

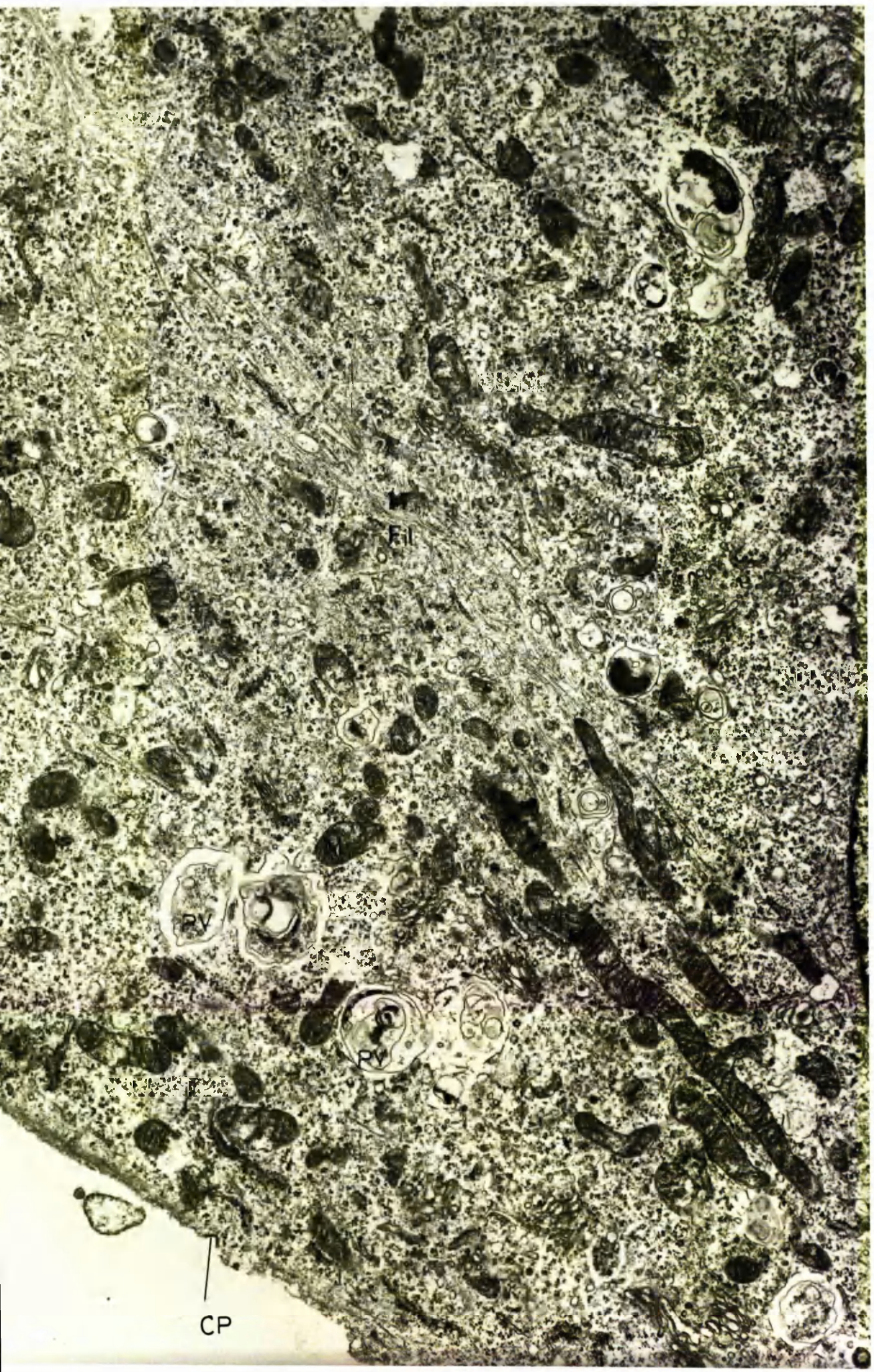

Fil
PV
PV
CP

Plate 8b.

Transmission electron micrograph of endothelial - like cells showing Weibel - Palade - like (WP) inclusions. Magnification 25,000 x. Scale bar = 1µm

Plate 9.

Transmission electron micrograph of endothelial - like cells, to show general appearance of cells. N = nucleus, M = mitochondria, PV = phagocytotic (endocytotic) vesicles, MP = microprocesses (psuedopodia - like projections) which appear to be all over the cell surface. Magnification = 26,500 x. Scale bar = 1μm.

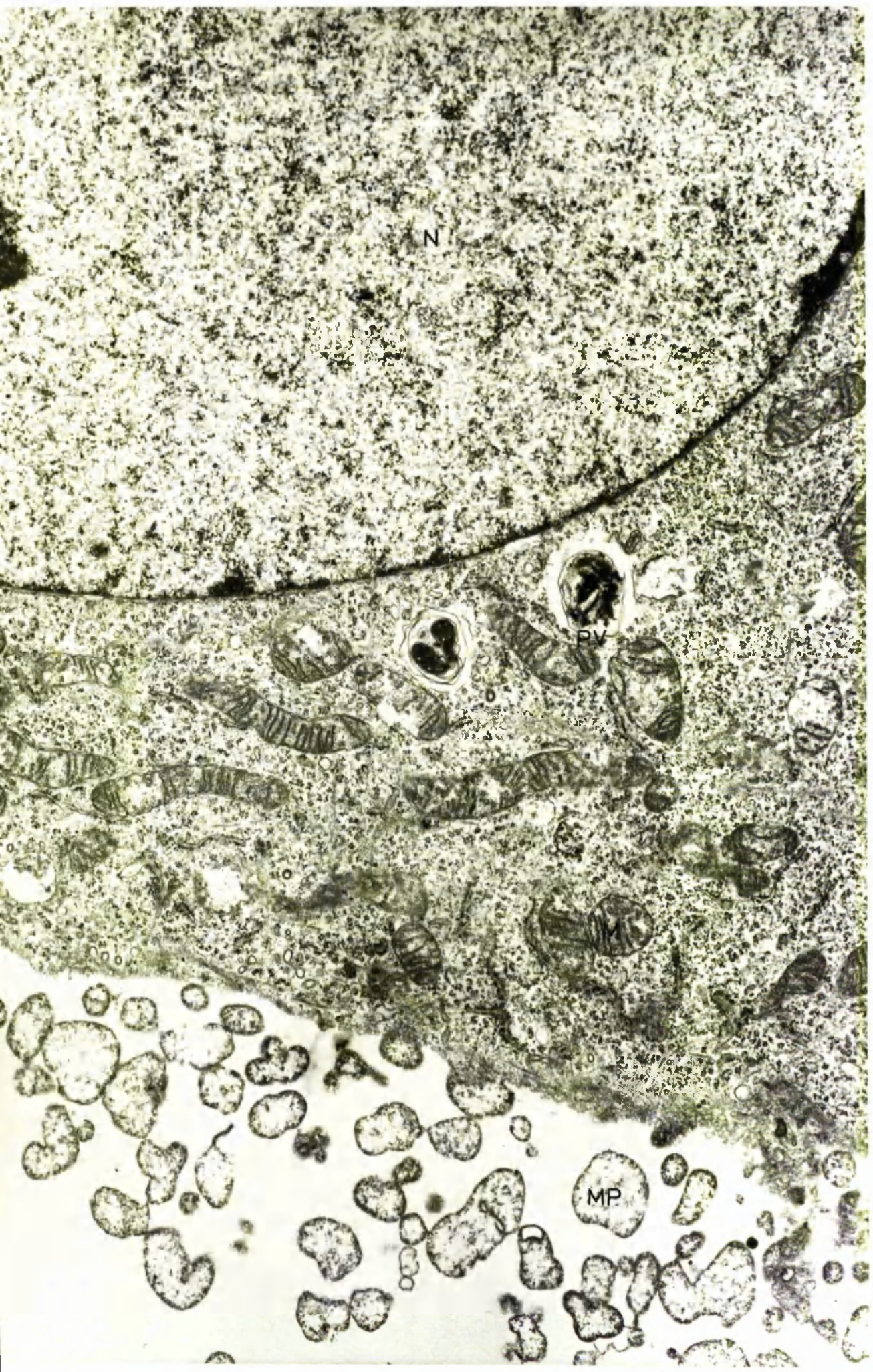

N
PV
M
MP

Plate 10.

As previous, showing microtubules (MT) microfilaments (MF), and mitochondria (M). Magnification 72,000 x. Scale bar = 1µm.

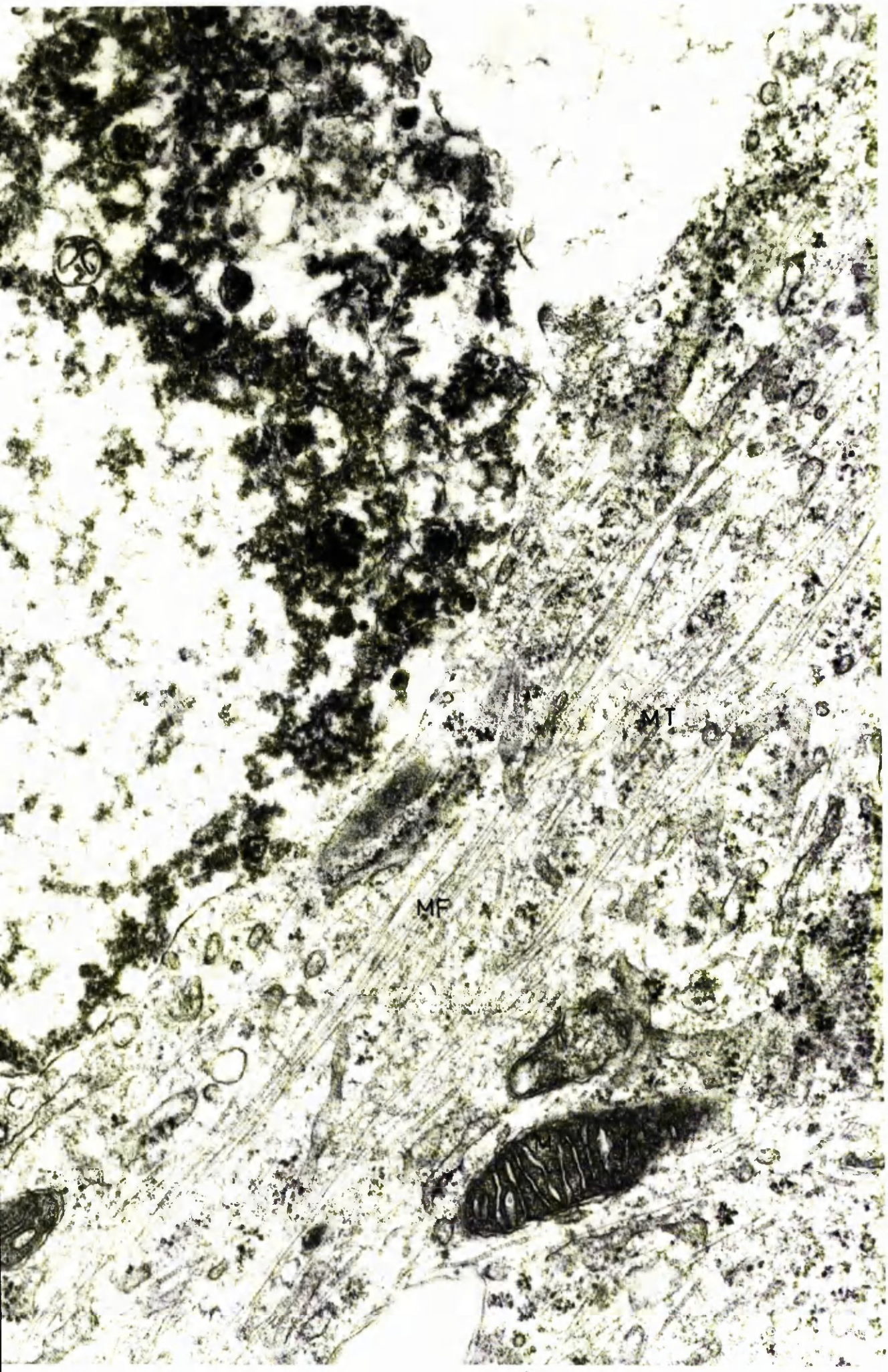

MT
MF

Plate 11.

As previous, showing intermediate filament bundles (Int. Fil.) which have been shown by SDS-PAGE techniques to be vimentin, and mitochondria (M). Magnification 72,000 x. Scale bar = 1μm

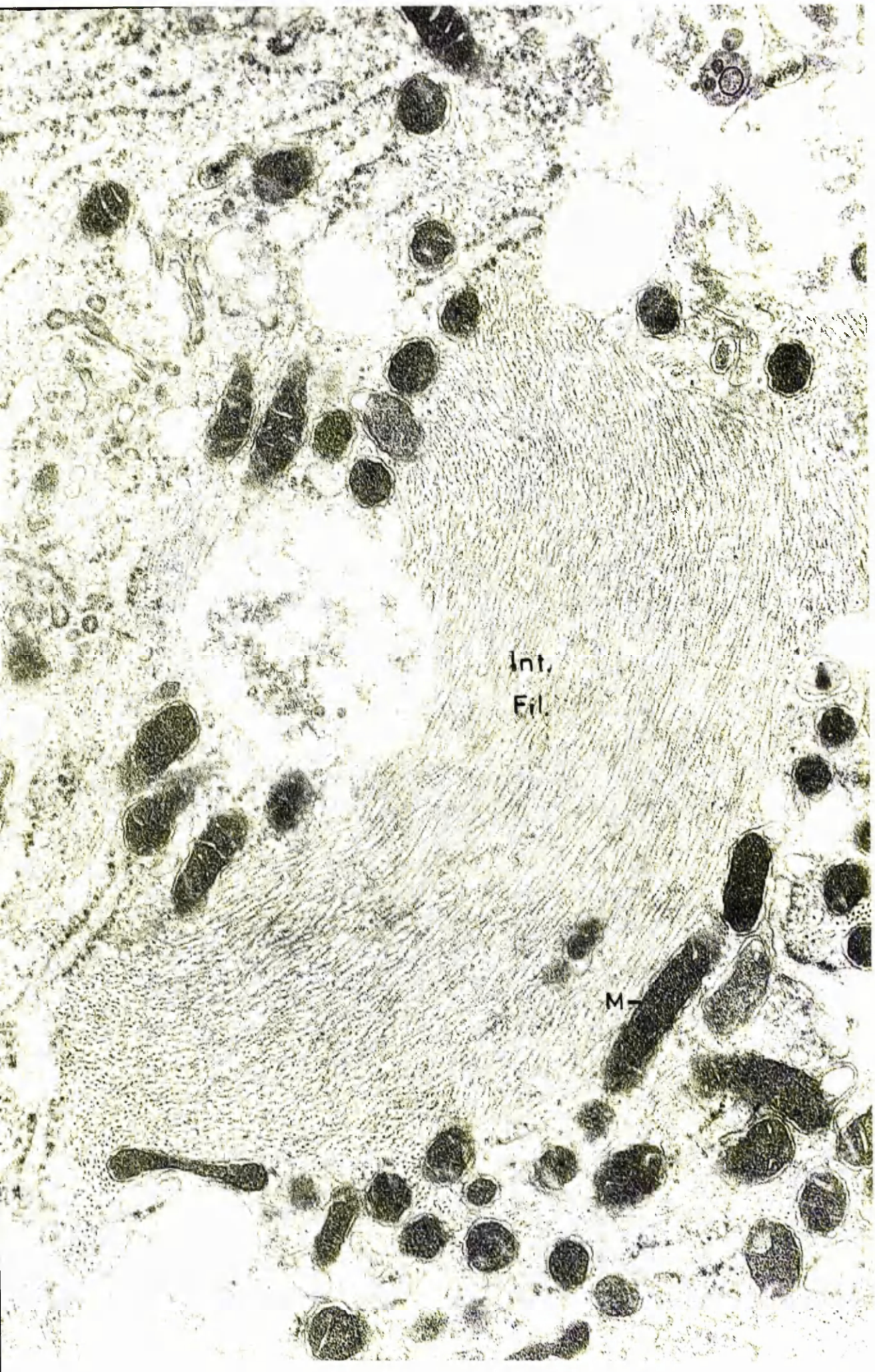
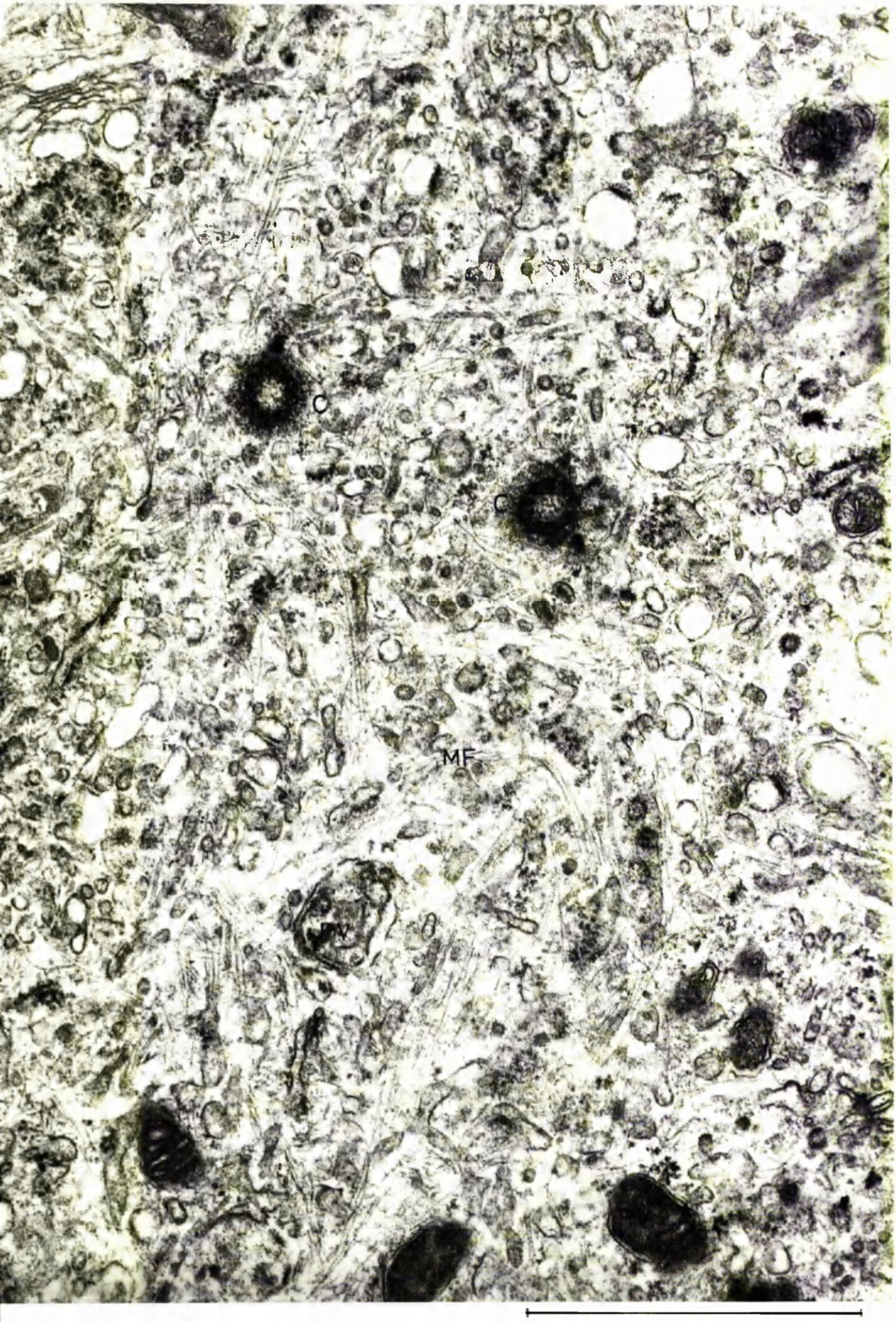

Int.
Fil.
M

Plate 12.

Final transmission electron micrograph of endothelial - like cells showing centrioles (C), microfilaments (MF) and phagocytotic (endocytotic) vesicles (PV). Magnification 72,000 x. Scale bar = 1μm.


C
C
MF

Conclusions

The lack of cytokeratin intermediate filament proteins in the endothelial - like cells correlates with the failure to find desmosomes in T.E.M. sections, since cytokeratins and desmosomes are associated in epithelial cells. This suggests that the cells are unlikely to be of an epithelial origin. The morphological appearance of the endothelial - like cells in the light and electron microscopes indicate that the cells are neither fibroblast - nor macrophage - like. The fact that perinuclear Factor VIII antigen was shown to be present in the cells, and angiotensin converting enzyme was released from them in high quantities strongly suggests that the cells are of an endothelial lineage.

CHAPTER 4. COMPLEMENT - MEDIATED CYTOTOXICITY AND IMMUNOFLUORESCENT LABELLING OF CELLS EXPRESSING CLASS I H-2 ANTIGENS.

4.1. Complement Mediated Cytotoxicity And Immunofluorescent Labelling Of Cells Expressing H-2 Antigens.

A series of experiments were performed to determine whether a population of cells derived from a single strain were homologous with respect to expression of a particular H-2 allele. The cell type used was the endothelial - like cells discussed in the previous chapter, and these were reacted with monoclonal antibodies against either K^k or D^k alleles. Preliminary experiments were performed to establish that the monoclonal antibodies were in fact complement fixing. The results of one such series of experiments are presented in Table 2 .

Coverslip cultures were prepared and reacted with either the monoclonal 11.4.1 which is directed against K^k, or 15.5.5s which is directed against D^k. The mean percentage of lysed cells was calculated for each treatment. Controls were included where either antibody or complement had been omited. The results are presented in Table 3.

The results show that only those cells expressing the appropriate allele were lysed by these treatments. Interestingly, F_1 cells showed intermediate susceptibility where only one parental allele was the target for antibody.

In the experiments presented in this and the following chapters, numbers given in brackets are standard deviations.

Table 2.

Complement fixation tests.

Dilution Of Monoclonal Anti K^k Antibody.	Lysis Of Haemolysin-sens SRBC.
1/500	-
1/1000	-
1/1500	-
1/2000	*
1/2500	+
1/3000	+
1/4000	+
1/8000	+

* indicates variability in result between different experiments.

The dilution of haemolysin used in the above experiment was 1/2000 as obtained from a haemolysin assay. The results showed that 1ml of 1/1000 monoclonal anti K^k (11.4.1) bound to B10.A cells could sucessfully bind 50µl of complement so that the lysis of haemolysin sensitised SRBC was inhibited. When the monoclonal 11.4.1 was diluted up to 1/2000 the sensitised SRBC were lysed in this assay.

Table 3.

Complement - mediated cytotoxicity of endothelial-like cells expressing particular Class 1 antigens.

Cells	No.of Expts.	Allele at K	D	Mean % Lysis. Anti K^k	Anti D^k	No ab.	No Comp
B10.BR	12	k	k	72 (12)	69 (8)	17 (3)	6 (2)
B10.	8	b	b	1 (1)	4 (1)	3 (1)	1 (1)
B10.AKM	2	k	q	64 (5)	ND	5 (3)	3 (1)
B10.D2	3	d	d	5 (2)	ND	3 (1)	1 (1)
(B10.BR x B10.AKM) F_1	4	k	k/q	82 (6)	45 (5)	10 (3)	3 (2)
(B10.A x B10.AQR) F_1	8	k/q	d	49 (9)	ND	10 (6)	5 (3)

Percentage lysis was calculated by counting at least 250 cells, and in some cases up to 400 cells. Cell death was detected using either Trypan Blue or F.D.A.

Investigation Of Decrease In Cell Number After Treatment With Antibody And Complement.

The percentage of cells lysed after a single treatment with specific antibody and complement must be known before cells are subsequently fixed and stained for total cell count comparisons. Lysed cells remain stuck to the coverslips and cannot easily be distinguished morphologically from unaffected cells after fixation. To find the length of time taken for lysed cells to detach from the substrate, a video film was made of a culture after treatment. On subsequent analysis of the film, it was seen that some cells apparently unaffected immediately after treatment with antibody and complement rounded up and detached from the substrate soon after division. Detachment of cells from the substrate appears to be due to complement - mediated lysis. As a result of these observations, the following experiments were designed.

The first of these was to investigate the decrease in cell number with time after treatment with specific antibody and complement. The usual methods outlined above were used, then coverslips were reincubated in fresh culture medium for 24, 48, or 72 hours. They were fixed in formol saline and stained in Iron-Alum Haematoxylin and mounted in clearmount. Coverslips were counted on the Quantimet 720 Image Analysing Computer. The mean number of cells per low power field was calculated for each treatment. Each low power field had an area of 0.2867 mm^2. 10 fields were counted on each coverslip, and all coverslips were in duplicate. Therefore, means are calculated from 20 fields.

Table 4.

Demonstrating the decrease in cell density with time after treatment with antibody and complement.

Strain	Treatments	Time After Treatment. 24hrs	48hrs	72hrs
B10.AKM	Anti K^k + complement	200 (61)	155 (49)	399 (81)
	Control	218 (33)	250 (25)	326 (32)
B10.D2	Anti K^k + complement	173 (14)	230 (8)	294 (36)
	Control	195 (20)	235 (11)	280 (17)

Values given in the table show the mean number of cells per low power field (0.2867 mm^2), and the standard deviation. In each case ten fields were counted on duplicate coverslips. Cell counts were carried out using a Quantimet 720 Image Analysing computer. High standard deviations reflect the variability in cell density across the coverslips after treatment.

The greatest decrease in cell number relative to the controls was seen 48 hours after treatment of B10.AKM cells. Another strain type was examined over a longer time course to determine whether the same results would be obtained.

Table 5.

Demonstration of reduction in cell density after exposure to antibody and complement using B10.A endothelial - like cells.

	Hours After Treatment.			
Treatment.	24	48	96	192
Anti K^k + Complement	181 (36)	210 (25)	184 (33)	234 (50)
Control	280 (26)	405 (40)	534 (57)	570 (51)

All coverslips were prepared in duplicate. Results show mean number of cells per field with the standard deviation. Each mean has been calculated from values taken from 20 low power fields.

The results given in Table 5 show that the reduction in density of B10.A cells becomes apparent 48 hours after treatment. Interestingly, B10.A cells failed to recover to control levels as quickly as in other experiments (see tables 7, and 8). The reason for these discrepancies is unclear at present. Considering the results of the last two tables together it can be seen that different strains show different susceptibility and speed of recovery after these treatments.

Affected cells may in fact take more than 48 hours to detach from the substrate, but this may not correlate with the greatest decrease in cell number due to the continuing growth of the survivors.

The next question arising from this work concerned the survivors themselves. These cells had failed to respond to treatment. In the case of B10.D2 cells, this was presumably a result of the lack of the specific allele against which the antibody was directed. However, in the B10.AKM strain, all cells were of the K^k genotype, but some still survived. This could be due to the stage of growth of these cells. For instance, some of them may have been in the exponenetial growth phase in which H-2 expression was at a very low level and thus a series of repeated treatments would eventually lyse all the population. Conversely, it is possible that different cells express slightly different types of H-2 product as result of gene processing (Robertson, 1982; Eskinazi et al 1981). If this were the case, then repeated exposure to antibody and complement would not completely deplete the population. Another explanation could be that some of the cells had not sufficiently recovered from trypsinisation

procedures used in the preparation of coverslips and therefore were not displaying the optimal concentration of H-2 antigens on the cell surface for a reaction to take place. To investigate these possibilities, the following experiments were performed.

B10.A endothelial - like cells were seeded onto coverslips on day 0 at levels known to give semi - confluent cultures. They were then treated with antibody and complement on days 1, 2, or 3 following seeding. Two days after exposure to antibody and complement, the percentage of viable cells were calculated using Trypan Blue exclusion methods. Coverslips were fixed and stained and counted on the Quantimet Image Analysing Computer as previously described.

Table 6.

To test whether recovery from trypsinisation affected susceptibility of B10.A endothelial - like cells to complement - mediated cytotoxicity with monoclonal anti K^k.

	Number of days after trypsinisation.		
Treatment.	1	2	3
11.4.1 + C'	135 (17)	2 (2)	15 (5)
11.4.1 only	122 (13)	164 (30)	180 (20)
C' only	144 (27)	150 (25)	172 (10)
No 11.4.1 or C'	158 (35)	209 (23)	188 (26)
No treatment	106 (12)	149 (27)	ND.

Values shown above have been corrected to show mean number of live cells per field. This was done by determining the percentage of live cells in each culture before fixation using Trypan Blue exclusion methods. Total cells per field were obtained on the Quantimet, then the following correction was applied to each:

% Live cells before fix X Total no. of cells after fix.

= No. of live cells per field before fixation.

The results in Table 6 show that cells are least susceptible to treatment 24 hours after seeding onto coverslips. Consequently, for all experiments, coverslips were left at least 48 hours before use. This change in susceptibility was not related to the stage of growth since the cells were seeded at semi - confluent levels, and much larger increases in the cell numbers of controls would have occured if cells were in the exponential phase of growth. This finding has not been previously reported. It is unlikely that treatment with trypsin results in this reduced expression of H-2 K^k products, although it is possible that the mechanical manipulation on the cells during their preparation for seeding onto coverslips can affect cell surface expression of H-2 antigenicity. Experiments by McClay and Gooding (1978) have shown that trypsinisation alone does not affect H-2 expresssion.

The next experiment was designed to determine when the application of a second treatment would be most effective in reducing cell number. After one exposure to antibody and complement, coverslip cultures were washed and reincubated in growth medium for various lengths of time before a second treatment was applied. Two days after the second treatment cultures were fixed, stained and counted as usual. The results are given in Table 7.

Having determined the more effective time interval between treatments with antibody and complement, repeated exposures to antibody and complement were applied to various cultures to try to totally deplete the population. The results are given in Table 8.

Table 7.

To determine when the application of a second treatment will be more effective in killing cells.

All cells used were B10.A (H-2 K^d) endothelial- like cells. They were fixed 48 hours after the second treatment.

	Time Interval Between 1st and 2nd Treat.			
Treatment.	24hrs	48hrs	96hrs	168hrs
11.4.1 + C'	3 (1)	6 (2)	17 (7)	206 (42)
No 11.4.1.	90 (14)	121 (43)	160 (54)	174 (43)
2nd Treat.only	107 (38)	134 (36)	123 (39)	192 (30)
1st Treat.only	37 (20)	66 (30)	87 (20)	183 (44)
No 1st or 2nd Treatment.	130 (40)	170 (20)	190 (13)	200 (20)

Values given were corrected with viable counts taken immediately before fixation, to show the mean number of cells per 10 fields which had not been lysed by 48 hours after 2 treatments with antibody and complement. Standard deviations are given in brackets. The results show that the second treatment is more effective between 24 and 48 hours after the first.

Table 8.

Investigation of population depletion after repeated exposure to antibody and complement.

B10.A Endothelial - Like Cells.(K^k)

Treatment.	Days On Which Treatment Given. 1	1,2	1,2,3.
Anti K^k + C'	162 (36)	188 (52)	22 (5)
C' only	193 (37)	381 (38)	481 (68)
Anti K^k only	245 (42)	386 (61)	396 (47)
No ant. or C'	283 (45)	359 (48)	451 (36)
No treatment	300 (37)	340 (53)	452 (48)

B10.BR Endothelial - Like Cells.(K^k)

Treatment.	Days On Which Treatments Given. 1,2,3	1,3,5.
Anti K^k + C'	49 (20)	82 (27)
C' only	103 (26)	134 (32)
Anti K^k only	N.D.	125 (18)
No anti. or C'	131 (33)	N.D.
No treatment.	130 (22)	N.D.

(B10.AQR x B10.A) F_1 Endothelial - Like Cells.($K^{q/k}$)

Treatment.	Days On Which Treatment Given. 1,2,3.	1,2,3,5,6.	1,3,5,7,8.
Anti K^k + C'	87 (24)	60 (20)**	114 (26)*
C' only	128 (9)	126 (10)	106 (31)
Anti K^k only	N.D.	104 (25)	122 (32)*

(Means taken from 30 fields, 90 **, or 70 * fields)

The results of table 8 show that after 3 consecutive treatments, B10.BR cells seem to have become non-responsive to treatment. This implies that the population remaining did not express the specificity against which the monoclonal antibody (11.4.1) was directed. This result was confirmed using the [B10.A x B10.AQR] F_1 cells, where five treatments failed to result in any significant decrease from controls. Here the non-responsive population had probably increased in number due to reduced competition for substrate and nutrients. The results for the B10.A cells showed a much greater decrease than the others after treatment, perhaps indicating a more homologous population.

Immunofluorescent Labelling Of H-2 Antigens.

These experiments were designed to determine whether the heterogeneity of H-2K^k expression seen in the above could also be observed by immunofluorescence techniques. However, this method was found to be more difficult to quantify. The positive results recorded in Table 9 were those cells which showed greater fluorescence than the negative control (B10.AQR, ie. K^q). Differences in results could not be attributed to the methods of preparation of coverslip cultures since all had been seeded onto coverslips three days before treatment, and had received tha same media and handling throughout their preparation.

Table 9.

Immunofluorescent labelling of H-2 antigens on endothelial - like cells using monoclonal 11.4.1 anti K^k and rabbit anti mouse - TRITC.

To Demonstrate Specificity Of Labelling.

Strain	Allele at K	PBS only.	Fluorescence with RAM-TRITC + Anti K	No Anti K
B10.BR	k	-	+	-
B10.AQR	q	-	-	-
[B10.A x B10.AQR] F_1	k/q	-	65% -	-

To Demonstrate Heterogeneity Of H-2K^k Expression.

Strain	Allele at K	% Fluorescence with anti K^k + RAM-TRITC
B10.AQR	q	4 (1)
B10.AKM	k	98 (2)
[B10.AQR x B10.A] F_1	q/k	59 (10)

The above results show that the F_1 cells show some heterogeneity of H-2K^k expression. B10.AKM cells did not demonstrate this phenomenon.

Conclusions.

From the above experiments I conclude the following;

After trypsinisation, cells should be left to recover for at least 48 hours before reacting with antibody and complement if maximal reduction in cell number is required.

There appears to be a delay after treatment with antibody and complement before some cells are lysed. It is possible that a sub - lethal hit is given to these cells during treatment leading to a disturbance of ionic fluxes across the membrane, which causes subsequent cytolysis. This finding has not previously been reported and may be a feature of the lung endothelial - like cells used in these experiments.

Some cells within a K^k population are not susceptible to treatment with the monoclonal antibody 11.4.1 anti K^k implying a heterogeneity of H-2 expression within a population.

B10.A cells appear to be the most susceptible to treatment with 11.4.1 anti K^k and complement. This may imply that different strains may vary in the proportion of a particular allele expressed. Interestingly , the F_1 cells did not show the same degree of susceptibility to treatment as the parental B10.A cells.

Immunofluorescent studies confirmed the results seen with complement - mediated cytotoxicity. Here, the B10.AKM cells were seen to express more of the appropriate K^k antigen than F_1 cells.

To summarise, the results presented in this section show that heterogeneity of cell surface expression of K^k products as detected by monoclonal antibodies is a real phenomenon.

CHAPTER 5. ADHESION OF PERITONEAL EXUDATE CELLS TO H-2D FRAGMENT - COATED COVERSLIPS.

5.1. Adhesion Of Peritoneal Exudate Cells To H-2D Fragment-Coated Coverslips.

H-2 fragments can be bound to chemically altered surfaces as previously discussed (page 53). The following experiments were designed to determine whether cells adhere and spread differentially to syngeneic and allogeneic fragment coated surfaces. These experiments were performed jointly with Mr. R. Gallagher, Dept. Of Cell Biology, Glasgow University.

Population Density Studies.

Preliminary experiments were performed to determine the optimal cell density to be used in this series of investigations. Heat - inactivated foetal calf serum (HIFCS) was added to chemically modified substrates as a control. This has the disadvantage that serum is known to inhibit cell adhesion (Curtis, 1965, 1967; Forrester et al, 1977). Peritoneal exudate cells (PEC's) were added in various concentrations to HIFCS coated substrates for 30 minutes at 37°C, then were fixed in formal saline, stained with Kenacid Blue and counted on the Quantimet 720 Image Analysing Computer. Values obtained (Table 10) were means taken from 20 low power fields (2 duplicate preparations, 10 fields per preparation, 1 field is 2.867×10^5 μm^2). Areas are given in picture point units (1 ppu = 0.5734 μm^2); some variation was encountered in the values obtained for cell areas as measured on different days. This was a result of the settings used on the Quantimet, and therefore preparations within an experiment were all read on the same day using the same settings. Thus, areas between experiments are not quantitatively comparable.

Table 10.

To determine the optimal concentration of PEC's for use in adhesion experiments.

10a. Variation in spread area with cell density.

Cell Conc./ml	Mean cell area (p.p.u.)
5×10^4	712 (25)
1×10^5	808 (57)
5×10^5	745 (8)
1×10^6	747 (30)
5×10^6	525 (43)

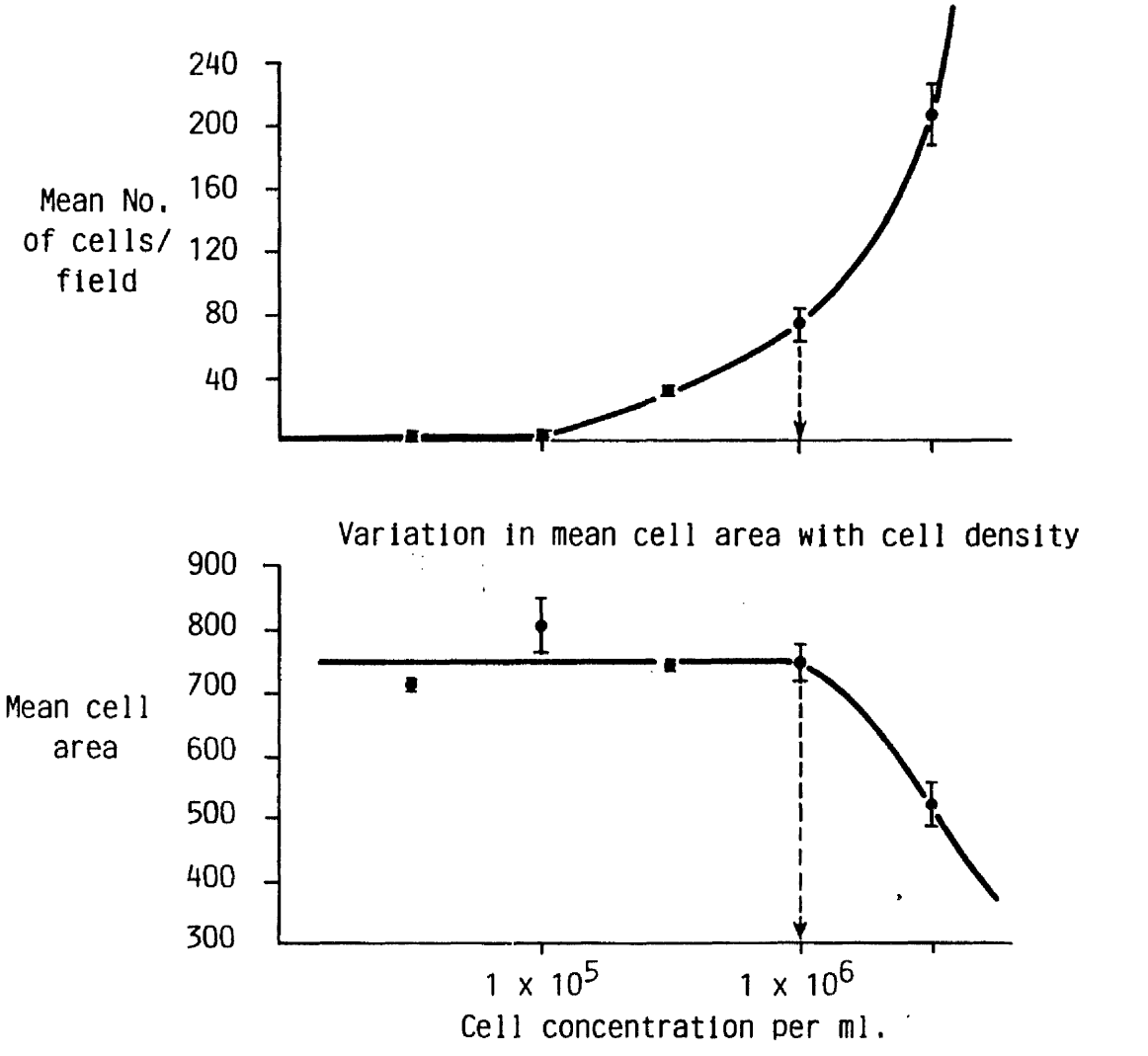
10b. Variation In Number Of Cells Per Field With Density.

Cell Conc./ml	Mean Cell Count Per $0.2867mm^2$
5×10^4	5 (1)
1×10^5	6 (2)
5×10^5	31 (3)
1×10^6	73 (15)
5×10^6	207 (27)

The cell concentration giving the optimal number of cells per field, and the mean cell area with no contact inhibition of spreading was approximately 1×10^6 cells / ml. (see fig 2). This was therefore used in further experiments.

FIG. 2.

Determining the optimal concentration of PEC's for use in adhesion experiments



The next experiment was designed to check that H-2 fragments were indeed being bound to chemically - modified surfaces. Monoclonal antibodies were used in an indirect immunofluorescence technique. The results are presented in Table 11. Fluorescence was only seen when monoclonal antibodies were bound to H-2D fragments attached to the substrate.

A further experiment was then designed to determine the optimal incubation time required for PEC adhesion and spreading on HIFCS - coated substrates. Cell counts and spread area on HIFCS - coated substrates were monitored over a period of 90 minutes to determine the incubation time to be used in these experiments. The results are shown in Table 12. One major disadvantage of this technique was the use of HIFCS in cell adhesion studies. Serum is known to contain factors which are inhibitory to cell adhesion. Therefore, serum was used, with reservations, only as a means of monitoring cell spread and count to protein - coated chemically - modified substrates. Perhaps purified serum proteins might have been a better choice for this system, but no doubt the cell count and spreading assays would vary according to the particular protein used. Furthermore, peritoneal cells are probably exposed to a mixture of serum proteins in vivo, some of which may adhere to the cell surface and confuse interpretation of results where a purified fraction is investigated. In later experiments, serum was not used in the incubation medium but proteins absorbed onto the cell surface in vivo may possibly have been present.

Table 11.

Detection of H-2 fragments on chemically modified surfaces.

H-2D Fragment.	Monoclonal antibody.	Fluorescence with RAM.-Fitc.
D^b	anti D^b	+
D^k	"	-
HIFCS	"	-
PBS	"	-
D^b	anti D^k	-
D^k	"	+
HIFCS	"	-
PBS	"	-

The monoclonal antibodies used in these experiments were 15.5.5s (anti D^b) and 141.34 (anti D^k).

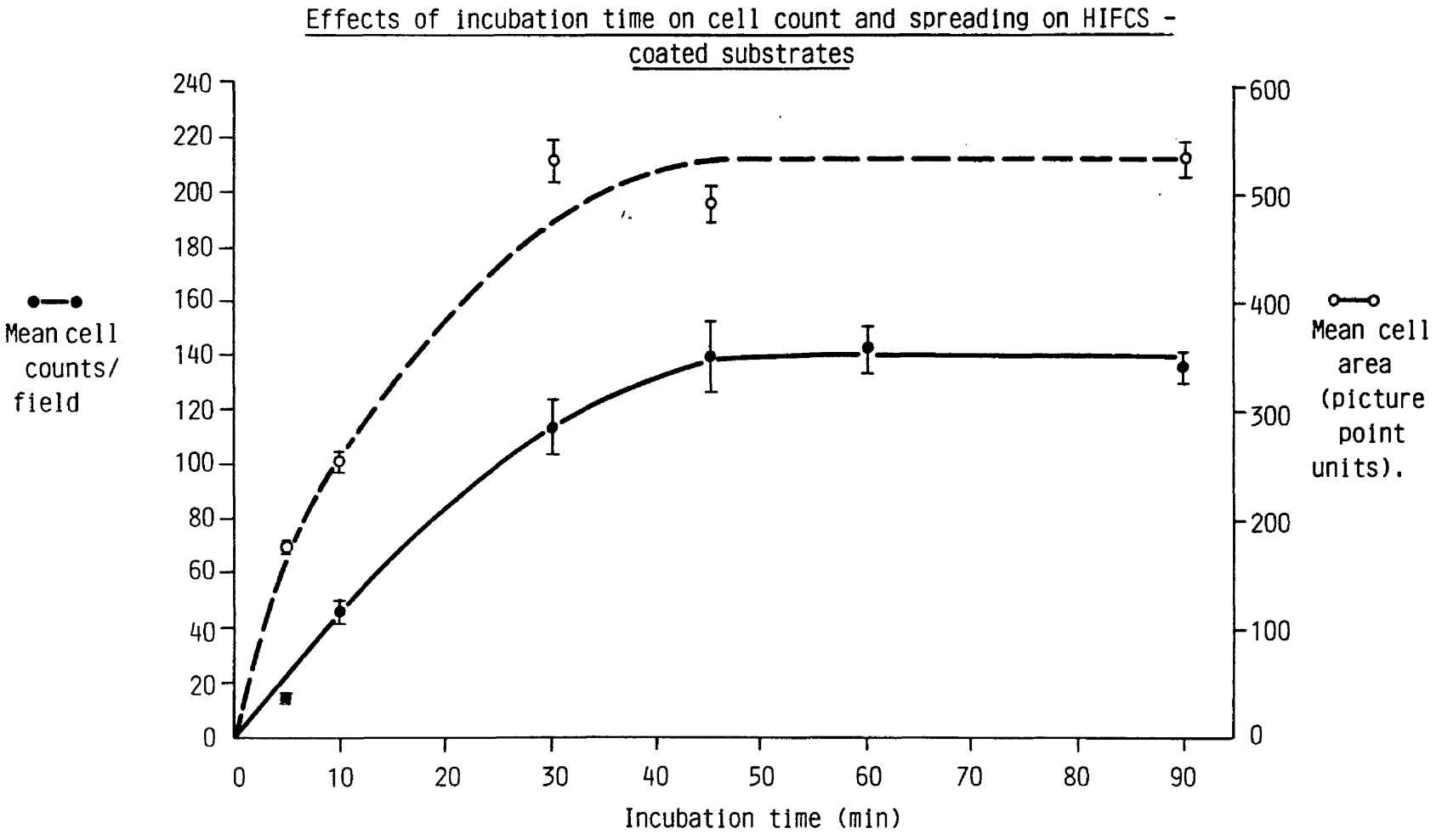
Table 12.

Effects of incubation time on cell count and spreading on HIFCS - coated substrates.

Inc. Time.	Mean No. Cells /field*	Mean Cell Area.(ppu)
5 mins	16 (2)	177 (4)
10 mins	47 (7)	254 (23)
30 mins	113 (11)	531 (40)
45 mins	140 (13)	493 (37)
60 mins	142 (9)	N.D.
90 mins	138 (7)	538 (31)

* Each field is 0.2867 mm2

FIG. 3.


Effects of incubation time on cell count and spreading on HIFCS - coated substrates
Mean cell counts/ field
Mean cell area (picture point units).
Incubation time (min)
0
20
40
60
80
100
120
140
160
180
200
220
240
0
100
200
300
400
500
600
0
10
20
30
40
50
60
70
80
90

The results given in Table 11 show that monoclnal antibodies will only bind to chemically modified surfaces when the particular H-2D fragment against which they are directed is present. This implies that H-2D fragments are bound to chemically modified surfaces in an antigenically recognisable form.

Table 12 shows that an incubation time of 30 mins allows most of the adherent population of PEC's to adhere and spread maximally for the purposes of these investigations. All further experiments were conducted using a 30 minute incubation time.

Interference Reflection Microscopy.

Syngeneic and allogeneic combinations of cells and substrate were examined using interference reflection microscopy to determine whether or not the number and/or distribution of adhesion plaques differed between combinations. No obvious differences were seen by this method. Quantitative comparisons of spread area and cell counts were subsequently made of the same preparations.

Comparison Of Spread Area And Cell Counts On Different Substrates.

Coverslips were fixed and stained with Kenacid Blue or Iron - Alum Haematoxylin and were examined on the Quantimet 720 Image Analysing Computer. Table 13 shows a comparison of cell counts on different substrates. Table 14 shows both cell counts and mean cell area of PEC's on different substrates. Areas were determined in picture point units as previously mentioned. Mean area per cell was obtained by dividing the total area covered with cells by the number of cells per field.

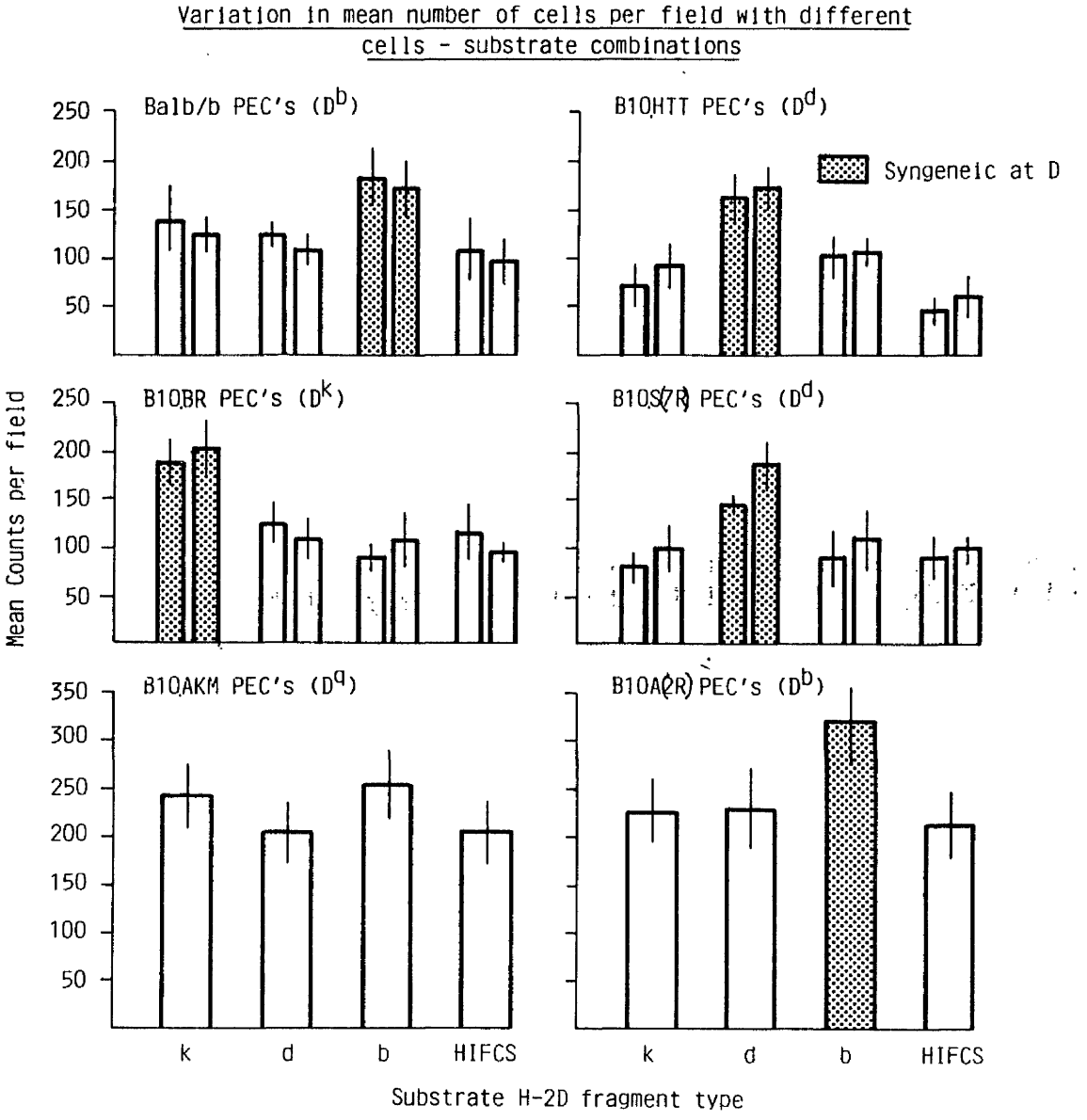
Table 13.

To show the variation in mean number of cells per $0.2867\ mm^2$ with different cell - substrate combinations.

Strain + H-2D Type.		Substrate H-2D Fragment Type. k	d	b	HIFCS
Balb/b	b	139 (28)	123 (14)	185 (32)	106 (30)
		125 (20)	111 (14)	175 (29)	98 (22)
B10.HTT	d	72 (26)	163 (27)	107 (20)	48 (20)
		95 (23)	173 (18)	104 (25)	62 (12)
B10.BR	k	187 (25)	124 (18)	90 (13)	112 (21)
		201 (28)	109 (24)	108 (27)	95 (9)
B10.S(7R)	d	83 (14)	148 (12)	93 (28)	93 (22)
		102 (23)	165 (21)	114 (30)	101 (17)
B10.AKM	q	244 (32)	205 (31)	255 (35)	207 (32)
B10.A(2R)	b	229 (33)	230 (39)	321 (36)	215 (33)

Where more than one result is presented for a particular strain and substrate type, results from single experiments are shown horizontally across the table. Standard deviations are given in brackets. These results are depicted in the histogram overleaf (fig. 4).

FIG. 4.


Variation in mean number of cells per field with different cells - substrate combinations
Balb/b PEC's (D^b)
B10.HTT PEC's (D^d)
Syngeneic at D
B10.BR PEC's (D^k)
B10.S(7R) PEC's (D^d)
B10.AKM PEC's (D^q)
B10.A(2R) PEC's (D^b)
Mean Counts per field
250
200
150
100
50
350
300
k
d
b
HIFCS
Substrate H-2D fragment type

Although values vary slightly from one experiment to another, the same increases in cell number are seen between treatments, i.e: H-2D syngeneic interactions always produce higher counts. This is more apparent from the histogram of these results (fig.4). To further investigate this finding, and also to determine whether there was an effect of H-2D substrate type on mean cell area, a further experiment was done. The two strains used were B10.BR ($H\text{-}2D^{k}$) and Balb/b ($H\text{-}2D^{b}$). The same experimental format was followed as for the previous work. The results are presented in Table 14. Again, it was found that cells adhere in greater numbers to syngeneic fragments, but their spread area was reduced as compared to allogeneic combinations. These results are given in fig. 5.

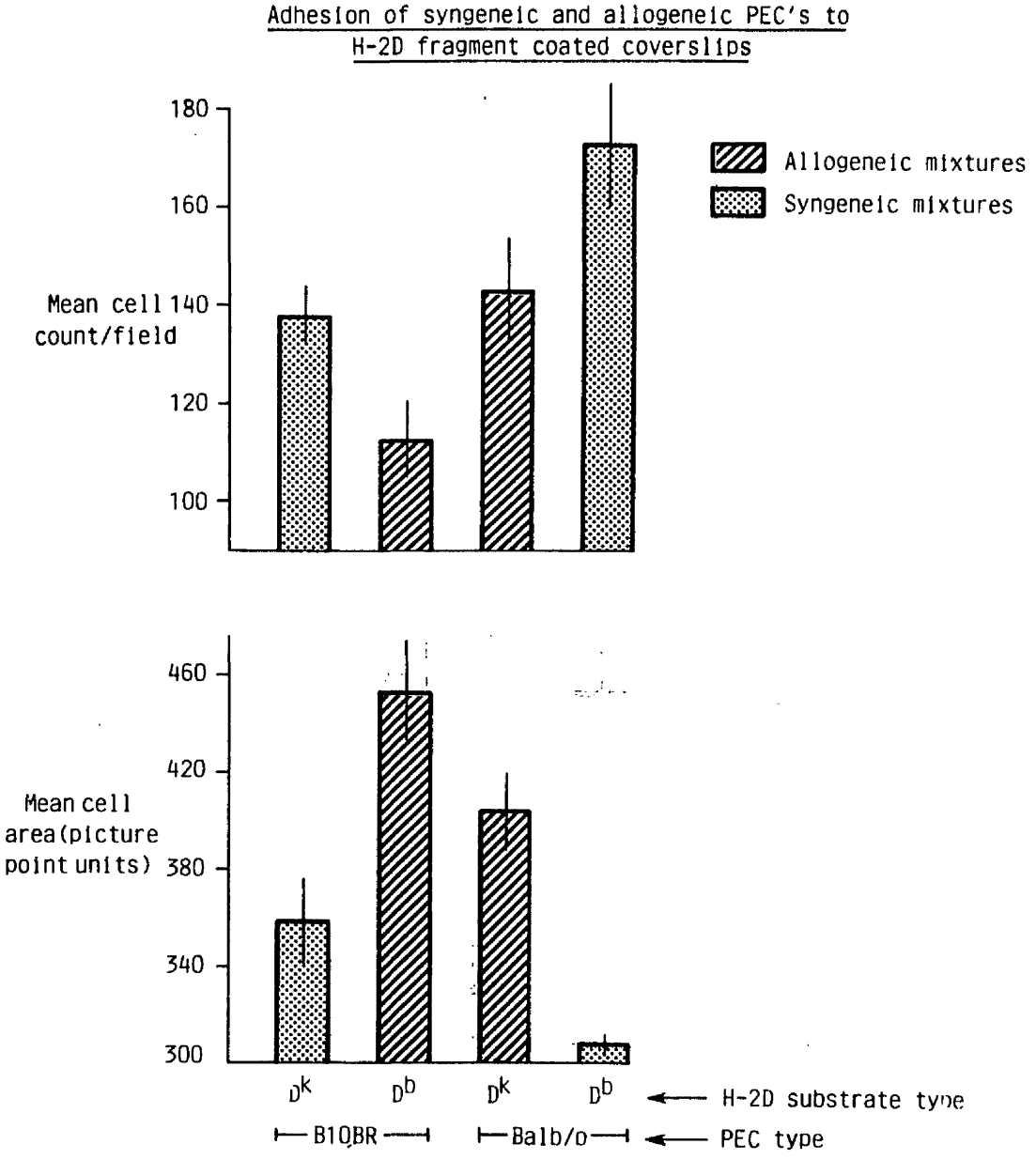
Table 14.

To investigate the adhesion of syngeneic and allogeneic PEC's to H-2D fragment - coated coverslips.

Strain Type	H-2D Type	Substrate	Mean count	Mean cell area.
B10.BR	k	D^k	138 (5)	359 (18)
		D^b	113 (8)	453 (21)
Balb/b	b	D^k	143 (11)	404 (16)
		D^b	173 (13)	308 (4)

A histogram of these results is presented in Fig 5.

FIG. 5.


Adhesion of syngeneic and allogeneic PEC's to H-2D fragment coated coverslips
Mean cell count/field
180
160
140
120
100
Allogeneic mixtures
Syngeneic mixtures
Mean cell area (picture point units)
460
420
380
340
300
D^k
D^b
D^k
D^b
H-2D substrate type
B10BR
Balb/c
PEC type

Conclusions.

The results presented in this chapter suggest that PEC's adhere to syngeneic substrates in greater numbers than to allogeneic substrates, but their spread areas on syngeneic substrates are reduced as compared to allogeneic combinations. It is unlikely that the differences seen in spread area are due to contact inhibition as the cells were not close enough to physically touch (as seen by direct observation of the preparations). Furthermore, preliminary experiments showed that contact inhibition of cell spreading did not occur at the cell concentrations used (see fig 2 + 3).

Mr. R. Gallagher has also examined the ability of activated and unactivated PEC's to adhere to fragment coated substrates, and has found no significant difference between the two.

CHAPTER 6. EFFECTS OF CONDITIONED MEDIUM ON THE NUMBER AND SPREAD AREA OF PERITONEAL CELLS ON GLASS SUBSTRATES.

6.1. Effects Of Conditioned Medium On The Number And Spread Area Of Peritoneal Exudate Cells On Glass Substrates.

Introduction.

Results presented in the last chapter showed that cells adhere differentially to syngeneic and allogeneic H-2D - coated substrates. A series of experiments were performed to determine whether syngeneic or allogeneic H-2 fragments in solution could react with cells and alter their adhesiveness to glass substrates. Cell counts per low power field (0.2867 mm^2 in area) and mean cell areas in picture point units (1 ppu = 0.5734 μm^2) were compared for different treatments. Means were calculated from 20 field (10 fields per duplicate preparation).

Emerson et al (1980) have previously shown that cells shed H-2 products into the culture medium. Though they found lipid associated material, Curtis and Davies (1982) reported soluble H-2 fragments in medium obtained from lymphocytes. The conditioned medium mentioned in this section was separated from living cells and passed through a series of Amicon filters to separate it into three fractions according to molecular weight. These were as follows:

Fraction 1 (f1) - < 10 K Daltons.

Fraction 2 (f2) - 10 - 35 K Daltons.

Fraction 3 (f3) - > 35 K Daltons.

The fractionation is known to be rather impure, as will be demonstrated later using SDS-PAGE techniques. However, fractions 2 and 3 contained more of the high molecular weight proteins and I felt that H-2 fragments shed from the cell surface

would be predominantly in fraction 2. This fraction was used in the experiments presented here, except where indicated. Conditioned media were obtained from the pooled supernatants of 1×10^7 spleen cells and / or PEC's which had been cultured in Hams F10 + 2.5% HIFCS + ITS (see general tissue culture methods, p.54). These were diluted in Hanks Hepes Saline to give concentrations of 1mg total protein per ml.

All the results in this section were tested for significance at the $P < 0.05$ level of the standard t test. Standard deviations are given in brackets in the tables, and are shown on the histograms.

The first experiment investigated the effects of various types of conditioned media on mean count and spread area per cell. B10.BR PEC's were incubated in spleen cell and / or PEC conditioned medium for 30 minutes at 37°C in rotatory cultures. After rigorous washing, the cells were allowed to adhere to clean glass coverslips for 30 minutes at 37°C. They were fixed, stained, and counted on the Quantimet 720 Image Analysing computer. Mean counts were calculated by counting the number of cells in each of 20 fields as usual. The field size was 0.2867 mm^2. The results are given in Table 15 and fig. 6.

Table 15.

Effects of fraction 2 (f2) conditioned media from different mouse strains on mean cell number and spread area (measured in picture point units) of B10.BR ($H\text{-}2^k$) PEC's.

B10.BR PEC'S + 3 day spleen + PEC conditioned medium.

Cond. Med.	Haplotype	Mean Count per 0.29mm^2	Mean Area/Cell
Uncond.	-	18 (5)	132 (17)
		20 (9)	N.D.
C3H	kkkkkkkkk	19 (9)	150 (13)
		19 (5)	N.D.
Balb/c	ddddddddd	38 (16)	181 (9)
		37 (8)	N.D.
B10.A	kkkkkdddd	20 (8)	155 (16)
		23 (8)	N.D.

B10.BR PEC's + 3 day spleen cell conditioned medium.

Uncond.	-	16 (3)	137 (8)
B10.BR	kkkkkkkkk	20 (5)	162 (15)
B10.A(4R)	kkbbbbbbb	18 (4)	176 (12)

B10.BR PEC's + 3 day PEC conditioned medium.

Uncond	-	16 (4)	137 (8)
B10.BR	kkkkkkkkk	21 (5)	175 (25)
B10.A(4R)	kkbbbbbbb	24 (6)	186 (21)
B10.	bbbbbbbbb	37 (8)	299 (21)
B10.HTT	sssskkk?d	33 (6)	254 (24)
B10.S(7R)	sssssssd	38 (9)	242 (21)

These results are presented in fig. 6.

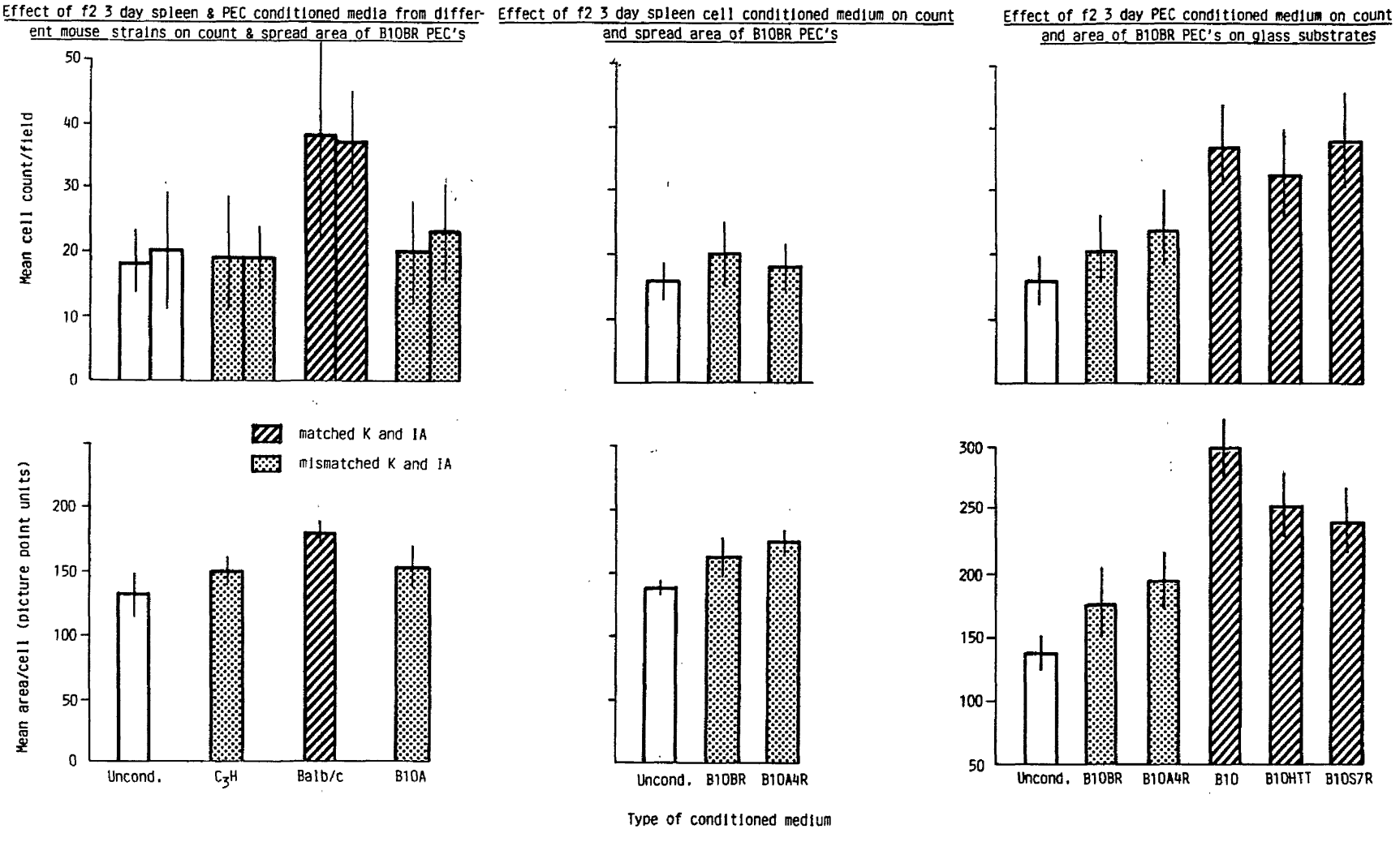

Effect of f2 3 day spleen & PEC conditioned media from different mouse strains on count & spread area of B10BR PEC's
Mean cell count/field
50
40
30
20
10
0
matched K and IA
mismatched K and IA
Mean area/cell (picture point units)
200
150
100
50
0
Uncond.
C3H
Balb/c
B10A
Effect of f2 3 day spleen cell conditioned medium on count and spread area of B10BR PEC's
Uncond.
B10BR
B10A4R
Effect of f2 3 day PEC conditioned medium on count and area of B10BR PEC's on glass substrates
300
250
200
150
100
50
Uncond.
B10BR
B10A4R
B10
B10HTT
B10S7R
Type of conditioned medium

FIG. 6.

The results given in Table 15 (fig. 6) show that when fraction 2 of conditioned medium is syngeneic at K and I-A loci, there is very little effect on the counts and spread area of B10.BR PEC's as compared to controls where the same cells had been incubated in fraction 2 of unconditioned medium. However, when the conditioned medium is allogeneic at these loci, there is an increase in both cell counts and spread area on clean glass substrates. Furthermore, spleen cell and / or PEC conditioned media of a given H-2 type had the same effects on cell count and spread area of a particular PEC type. These investigations were extended to B10.A(4R) and (B10.BR x B10.A(4R)) F_1 PEC's. Spleen and PEC conditioned media were used separately, and again the cell counts and spread areas were compared for each treatment. The results are given in Table 16 (fig. 7). Once again, cell counts per 0.29 mm^2 and spread cell areas were increased when conditioned media were allogeneic at K and I-A. Other locus differences did not seem to have the any effect on cell counts and spread area.

Table 16.

Effects of fraction 2 (f2) conditioned medium from different strains on mean cell count per 0.29 mm^2 and spreading.

B10.A(4R) PEC's + 3 day PEC conditioned medium.

Cond. Med.	Haplotype	Mean Count	Mean Area/cell (ppu)
Uncond.	-	10 (7)	174 (22)
B10.A(4R)	kkbbbbbbb	15 (6)	183 (21)
B10.BR	kkkkkkkkk	15 (7)	163 (24)
B10	bbbbbbbbb	33 (13)	242 (21)
B10.HTT	sssskkk?d	26 (5)	224 (20)
B10.S(7R)	ssssssssd	30 (9)	235 (15)

B10.A(4R) PEC's + 3 day spleen conditioned medium.

Uncond.	-	10 (7)	174 (22)
B10.A(4R)	kkbbbbbbb	10 (3)	165 (35)
B10.BR	kkkkkkkkk	14 (7)	177 (24)

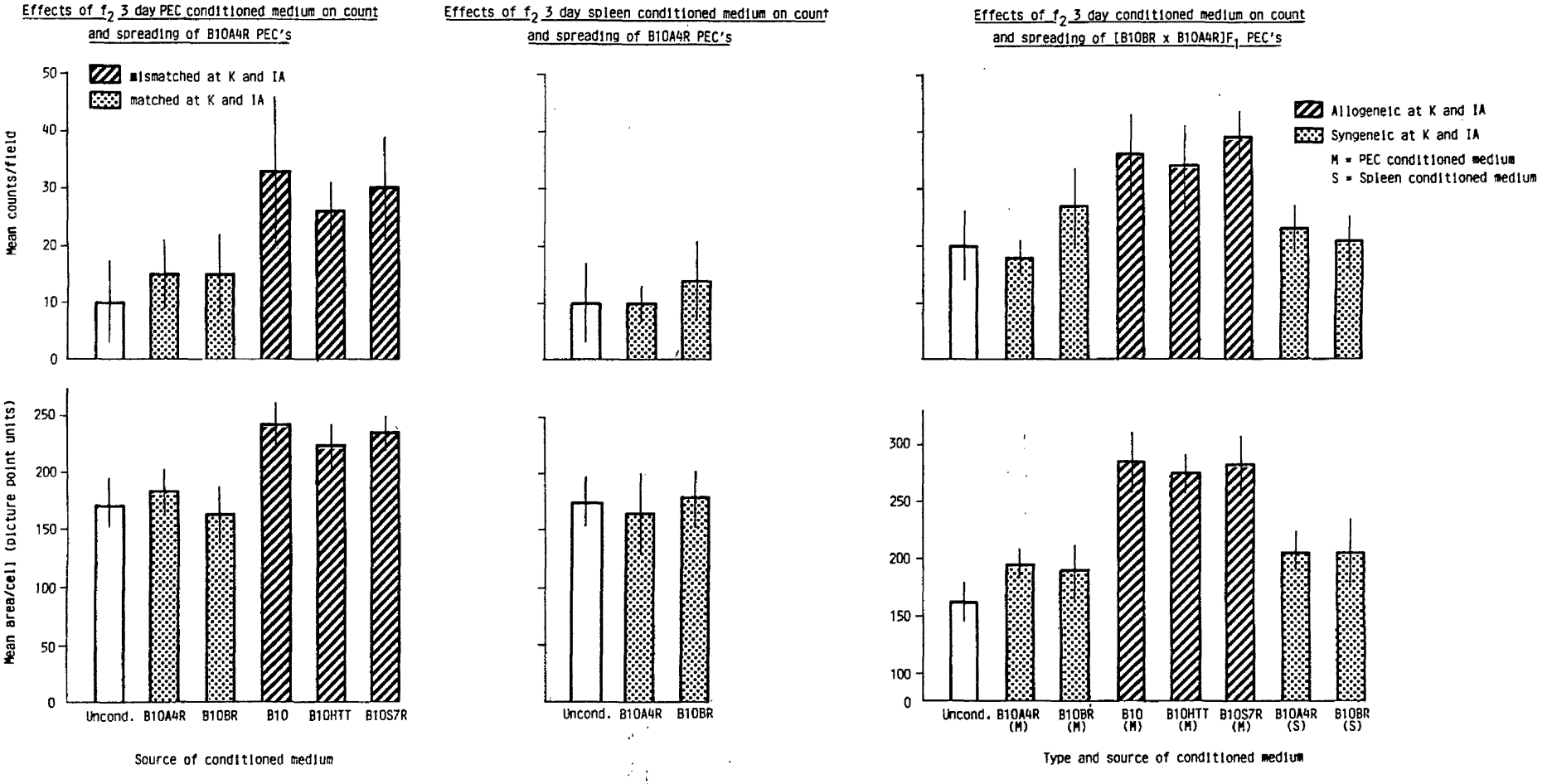
[B10.BR x B10.A(4R)] F_1 PEC's + 3 day conditioned medium

Uncond.		-	20 (6)	162 (17)
B10.A(4R)	(m)	kkbbbbbbb	18 (3)	196 (12)
B10.BR	(m)	kkkkkkkkk	27 (7)	190 (21)
B10	(m)	bbbbbbbbb	36 (7)	286 (26)
B10.HTT	(m)	sssskkk-d	34 (7)	275 (16)
B10.S(7R)	(m)	ssssssssd	39 (4)	283 (27)
B10.A(4R)	(s)	kkbbbbbbb	23 (4)	206 (17)
B10.BR	(s)	kkkkkkkkk	21 (5)	206 (29)

(m = PEC cond. med. s = spleen cond. med.)

These results are presented in fig 7.

FIG. 7.


Effects of f_2 3 day PEC conditioned medium on count and spreading of B10A4R PEC's
Mismatched at K and IA
matched at K and IA
Mean counts/field
Mean area/cell (picture point units)
Uncond. B10A4R B10BR B10 B10HTT B10S7R
Source of conditioned medium
Effects of f_2 3 day spleen conditioned medium on count and spreading of B10A4R PEC's
Uncond. B10A4R B10BR
Effects of f_2 3 day conditioned medium on count and spreading of [B10BR x B10A4R]F_1 PEC's
Allogeneic at K and IA
Syngeneic at K and IA
M = PEC conditioned medium
S = Spleen conditioned medium
Uncond. B10A4R (M) B10BR (M) B10 (M) B10HTT (M) B10S7R (M) B10A4R (S) B10BR (S)
Type and source of conditioned medium

The next experiment was done to determine whether mismatcing of PEC's and conditioned media at non - H-2 loci would have any effect on cell count and spread. It seems likely that other cell surface proteins are shed into the culture medium in addition to H-2 products. Therefore, as these would also be present in the conditioned media, they may enhance or reduce the effects of H-2 products. The results of these experiments are shown in Table 17. Four types of PEC's were examined, and in each case the effects of completely syngeneic conditioned media, unconditioned media and non - H-2 mismatched conditioned media on mean cell counts per field (0.29 mm^2) were compared. The results show that there is no evidence for any effect on cell counts due to non - H-2 products present in the conditioned medium.

Table 17.

Effects of f2 mixed PEC and spleen cell conditioned medium from different mouse strains on cell counts per 0.29 mm^2 when PEC's of various strain types are added to glass substrates.

PEC's	H-2 Type	Cond. Med.	H-2 Type.	Mean counts/ 0.29mm^2	
Balb/b	bbbbbbbbb	Uncond.	-	36	(14)
"	"	B10.A(3R)	bbbbkdddd	39	(12)
"	"	Balb/c	ddddddddd	72	(10)
"	"	Balb/b	bbbbbbbbb	37	(15)
B10.A	kkkkkdddd	Uncond.	-	27	(6)
"	"	C3H	kkkkkkkkk	30	(15)
"	"	Balb/c	ddddddddd	116	(14)
"	"	B10.A	kkkkkdddd	36	(17)
Balb/c	ddddddddd	Uncond.	-	24	(9)
"	"	B10.A	kkkkkdddd	43	(8)
"	"	Balb/c	ddddddddd	27	(8)
"	"	C3H	kkkkkkkkk	49	(5)
B10.HTT	sssskkk?d	Uncond.	-	25	(19)
"	"	B10.A(3R)	bbbbkdddd	46	(15)
"	"	B10.S(7R)	sssssssd	21	(8)
"	"	C3H	kkkkkkkkk	36	(11)
"	"	Balb/b	bbbbbbbbb	42	(11)
"	"	B10.A	kkkkkdddd	40	(20)
"	"	Balb/c	ddddddddd	42	(18)
"	"	B10.HTT	sssskkk?d	28	(11)

Mean areas were not counted in these experiments. These results are presented in fig. 8.

Effects of f_2 mixed 3 day spleen and PEC conditioned medium from different mouse strains on cell counts

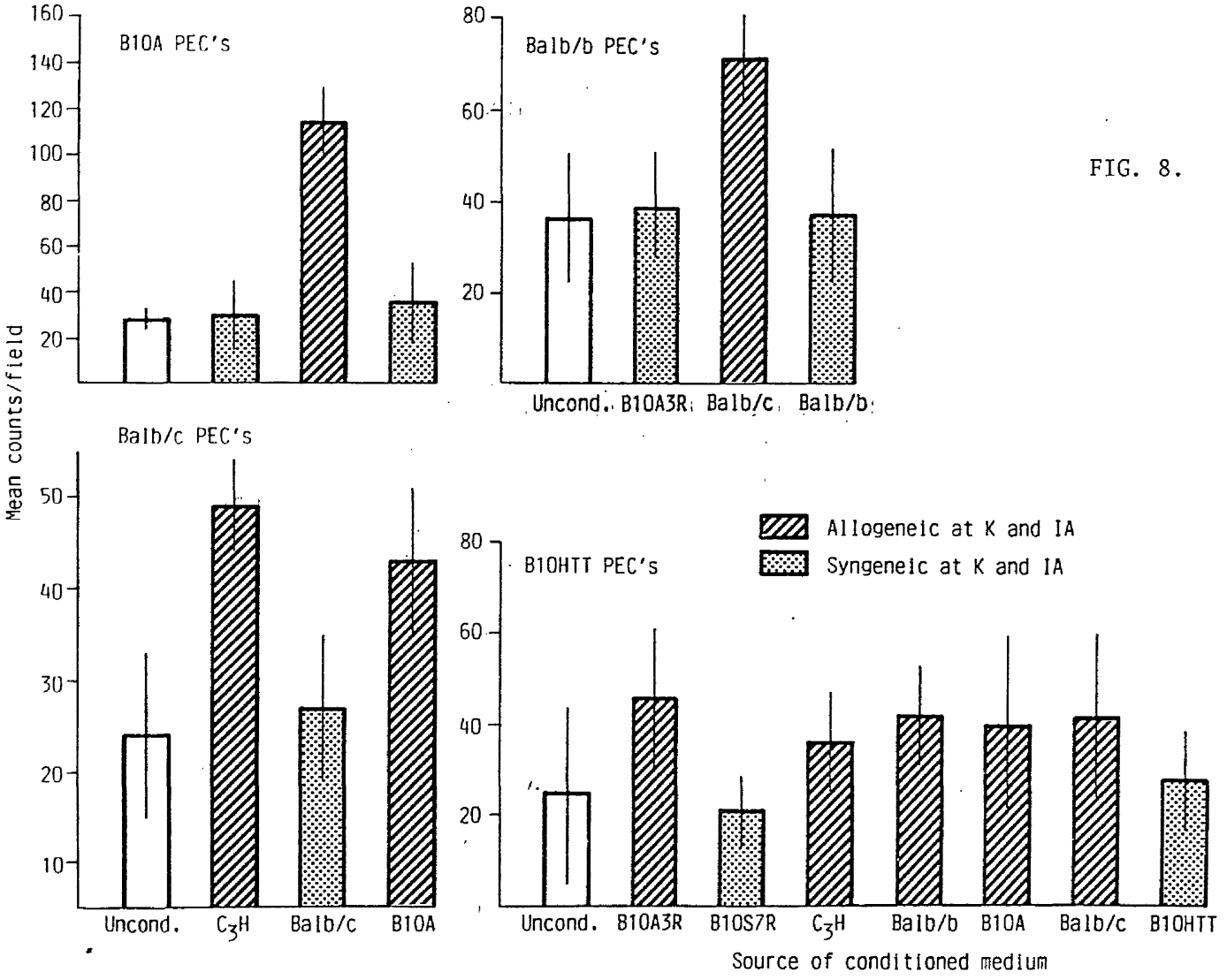


FIG. 8.

From the previous, it appears that H-2K and / or I-A fragments which are shed from cells during in vitro culturing can interact with PEC's which are allogeneic at these loci in rotatory cultures. As a result of this interaction, cells adhere to glass surfaces in greater numbers and also show increased spread area. To determine whether this phenomena may occur in other cell types, the same experimental format was used on cells of an endothelial - like lineage. The results (Table 18) show that endothelial - like cells behave in exactly the same manner as PEC's, i.e. they show increased cell counts and spread areas on glass substrates after preincubation in H-2K and / or I-A allogeneic conditioned medium.

Table 18.

Effect of f2 conditioned medium on the adhesion of B10 ($H\text{-}2^{b}$) endothelial - like cells to glass substrates.

Effect on cell counts per field.

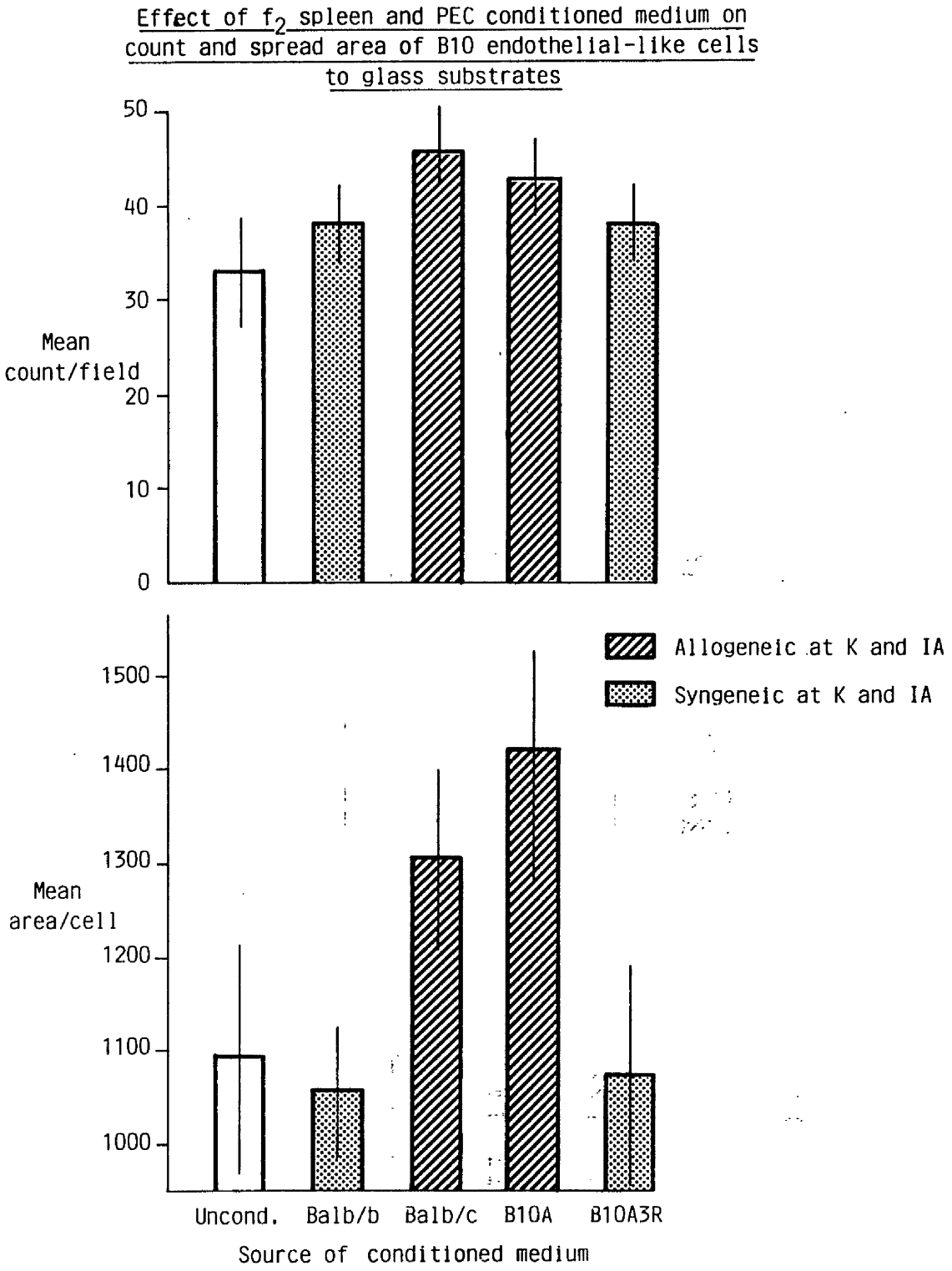
Cond.Med.	Haplotype	Mean Cell Counts / 0.29 mm^2
Uncond.	-	33 (6)
Balb/b	bbbbbbbbb	38 (4)
Balb/c	ddddddddd	46 (5)
B10.A	kkkkkdddd	43 (4)
B10.A(3R)	bbbbkdddd	38 (4)

Effect On Spread Cell Area.

Cond. Med.	Haplotype	Mean Spread Area (ppu)
Uncond.	-	1092 (128)
Balb/b	bbbbbbbbb	1058 (102)
Balb/c	ddddddddd	1303 (101)
B10.A	kkkkkdddd	1421 (147)
B10.A(3R)	bbbbkdddd	1074 (116)

These results are depicted in fig. 9.

FIG. 9.


Effect of f_2 spleen and PEC conditioned medium on count and spread area of B10 endothelial-like cells to glass substrates
Mean count/field
50
40
30
20
10
0
Mean area/cell
1500
1400
1300
1200
1100
1000
Allogeneic at K and IA
Syngeneic at K and IA
Uncond.
Balb/b
Balb/c
B10A
B10A3R
Source of conditioned medium

The previous experiments were all performed with fraction 2 of the conditioned medium, which is likely to contain most of the H-2 proucts shed from the cells. However, as previously stated, the fractionation by Amicon filtration was known to be rather impure. The other fractions were therefore tested to determine whether they would have similar effects to fraction 2. The results are shown in Table 19 (fig. 10). The three fractions of unconditioned medium were included to determine whether there was any factor present in them which had an effect on cell count and spread area. Fraction 3 of this unconditioned medium was shown to have a marked effect in reducing cell counts as compared to whole unconditioned medium. Effects on mean cell area were not significant at the P = 0.05% of the standard t test. Fraction 3 of H-2K and I-A allogeneic conditioned media resulted in the greatest increases in counts and spread areas of B10.BR PEC's.

SDS-PAGE analysis of the three fractions is shown in Plates 13, 14, and 15. This showed that fraction 1 contained very little protein as compared to the other two fractions, and fraction 3 contained the most. This is particularly apparent by considering the different amounts of the conditioned media added to the gels. In the case of fraction 1, 100µl were added, then 20 - 30 µl and 1µl for fractions 2 and 3 respectively. Silver staining of an SDS gel of fraction 2 showed that small amounts of a number of proteins were present, but these were not identified.

Table 19.

Effect of different fractions of spleen + PEC conditioned medium on mean cell number per 0.29 mm^2 and area (ppu) of B10.BR PEC's on glass substrates.

Cond. Med.	Fraction	Haplotype	Mean Count	Mean Area
Uncond.	1+2+3	-	42 (10)	303 (36)
"	1	-	38 (9)	323 (27)
"	2	-	26 (10)	260 (29)
"	3	-	18 (11)	271 (33)
Balb/b	1	bbbbbbbbb	28 (10)	349 (20)
"	2	"	39 (8)	282 (31)
"	3	"	66 (15)	363 (32)
C3H	1	kkkkkkkkk	29 (5)	297 (31)
"	2	"	25 (9)	288 (35)
"	3	"	21 (12)	291 (24)
B10.A	1	kkkkkdddd	27 (6)	267 (26)
"	3	"	26 (8)	280 (37)
B10.A(3R)	1	bbbbkdddd	28 (11)	283 (33)
"	2	"	45 (14)	329 (26)
"	3	"	48 (14)	339 (26)

These results are depicted in fig. 10.

FIG. 10.

Effect of different fractions of conditioned medium from spleen and PEC's on mean cell count and spread area of B10BR PEC's on glass substrates

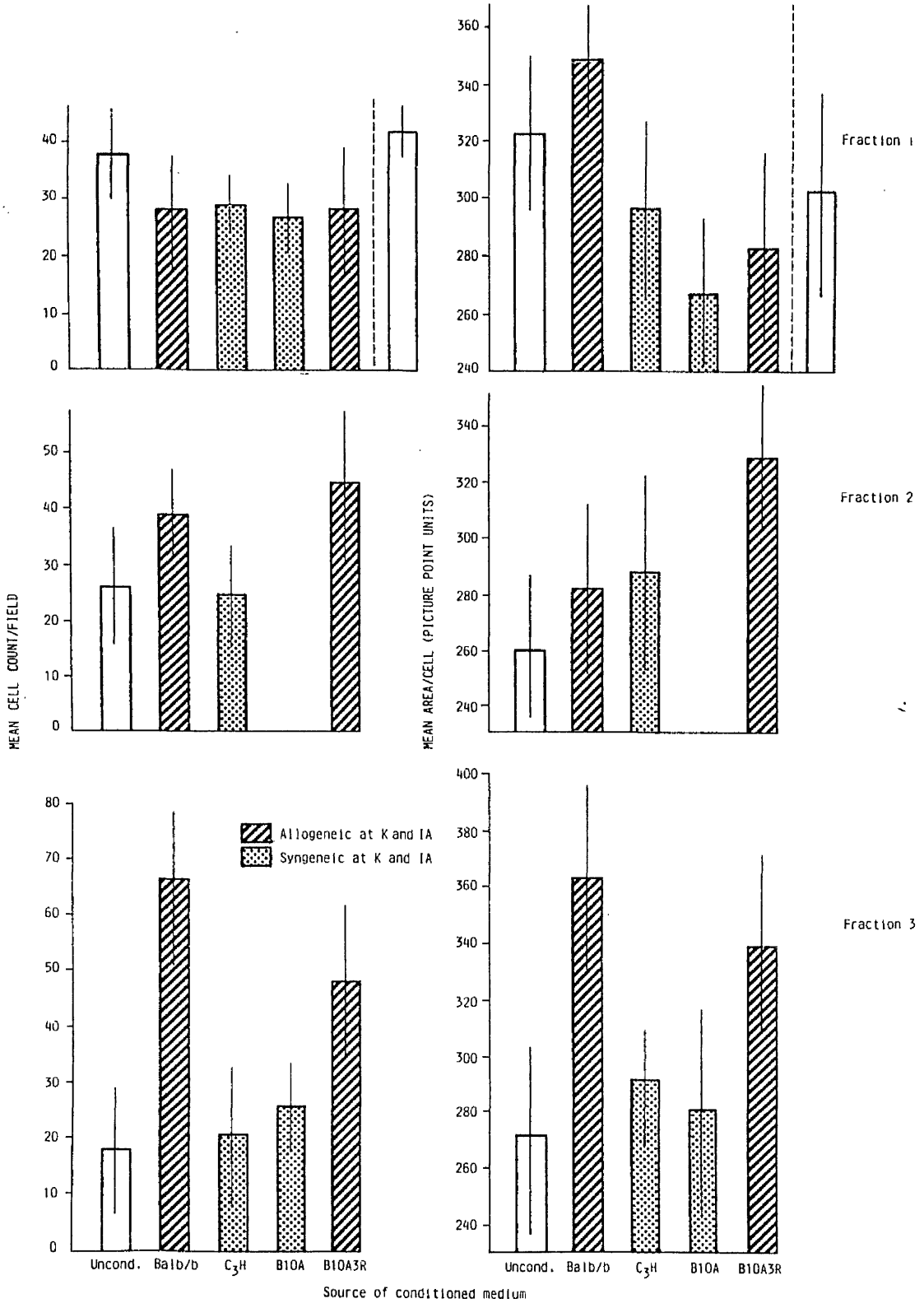


Plate 13.

SDS - polyacrylamide 6 - 10% gel stained with Kenacid Blue. This demonstrates that fraction 1 of conditioned medium from 72 hr cultures of PEC's and spleen cells contains very little protein, although some traces are seen in the 60 - 120 K Dalton range. The following preparations are shown on the gel:

20µg Ovalbumin, 45 K Dalton protein. (Tracks A and H - Ov).

100µl Fraction 1 of unconditioned Hams F10 + ITS + HIFCS (Track B)

100µl Fraction 1 of the following conditioned media obtained from PEC's and spleen cells;

Balb/b (Track C)

Balb/c (Track D)

C3H (Track E)

B10.A(3R) (Track F)

B10.A (Track G)

The bands seen in the f1 conditioned medium are also seen in f1 unconditioned medium, and are probably B.S.A. Protein concentrations are unknown and cannot be estimated accurately from this preparation. Molecular weight markers were obtained from an identical gel of standards run in parallel. These were as follows:

220	Fibronectin
120	Beta Galactosidase
95	Phosphorylase A
68	Bovine Serum Albumin
45	Ovalbumin
22	Concanavalin A

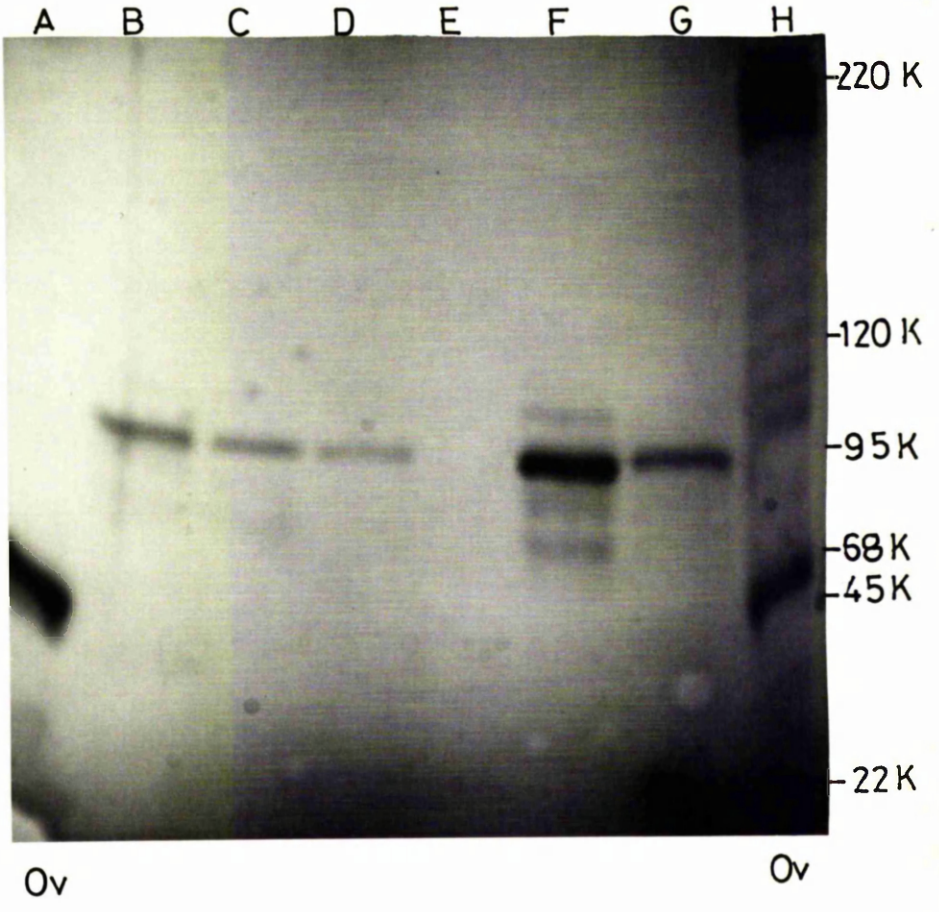

A
B
C
D
E
F
G
H
220 K
120 K
95 K
68 K
45 K
22 K
Ov
Ov

Plate 14.

SDS - polyacrylamide 6 - 10% gel stained with Kenacid Blue, demonstrating proteins present in fractions 2 (f2) and 3 (f3) of conditioned media. Note the differences in the amount of the two fractions loaded onto the gel. The following preparations are shown:

20µg Ovalbumin, 45 K Dalton protein (Tracks A and N - Ov).

20µl Fraction 2 of Hams F10 unconditioned medium (Track B).

1µl Fraction 3 " " " " " (Track H).

Conditioned media are in the following order;

	Fraction 2		Fraction 3	
	Amount (µl)	Position	Amount (µl)	Position
Balb/b	20	Track C	1	Track I
Balb/c	40	" D	1	" J
C3H	40	" E	1	" K
B10.A(3R)	20	" F	1	" L
B10.A	20	" G	1	" M

The bands seen between 60 and 100 K Daltons are probably Bovine Serum Albumin (BSA). Protein concentrations present cannot be accurately estimated from the above. Molecular weight markers were obtained from an identical gel of standards run in parallel. For list of standards, see previous gel.

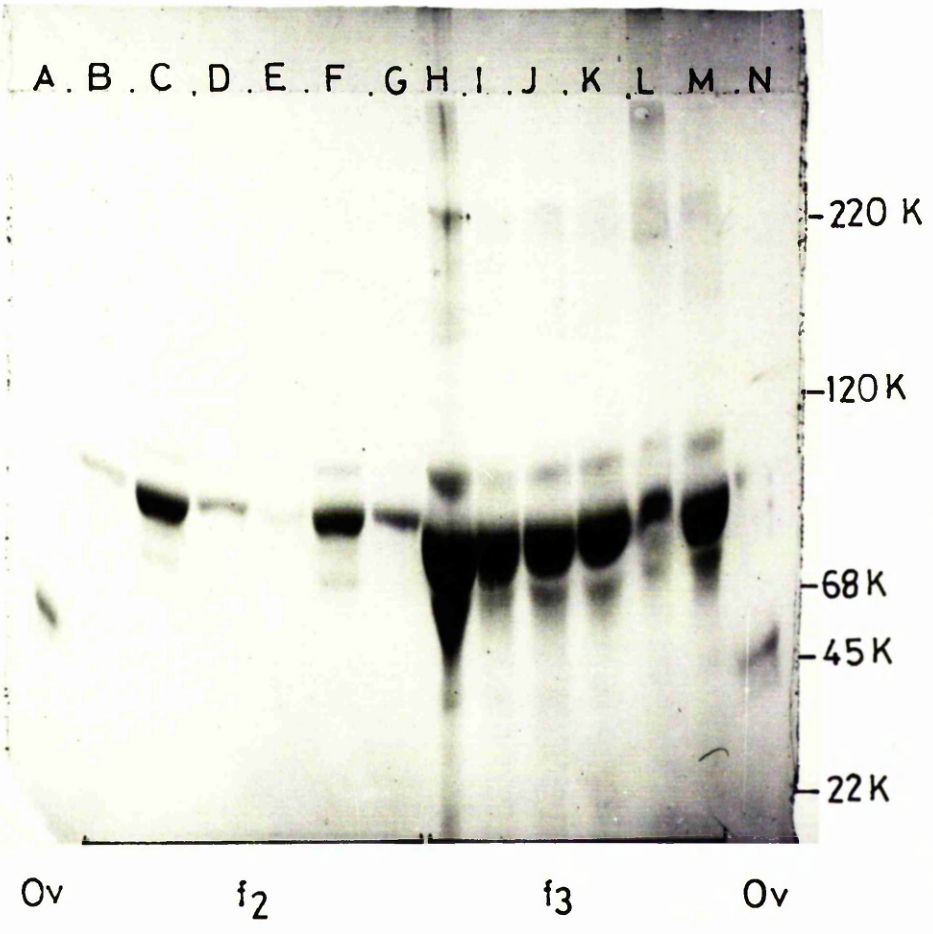

A B C D E F G H I J K L M N
220 K
120 K
68 K
45 K
22 K
Ov
f2
f3
Ov

Plate 15.

SDS - polyacrylamide 6 - 10% silver stained gel of 72 hr f2 conditioned media from spleen and PEC cells containing the following preparations:

20µg Ovalbumin. 45 K Dalton (Tracks A and J - Ov)

20µl Fraction 2 of Hams F10 + HIFCS + ITS unconditioned medium (Track B)

20µl Balb/b f2 conditioned medium (Track C)

20µl Balb/c " " " (" D)

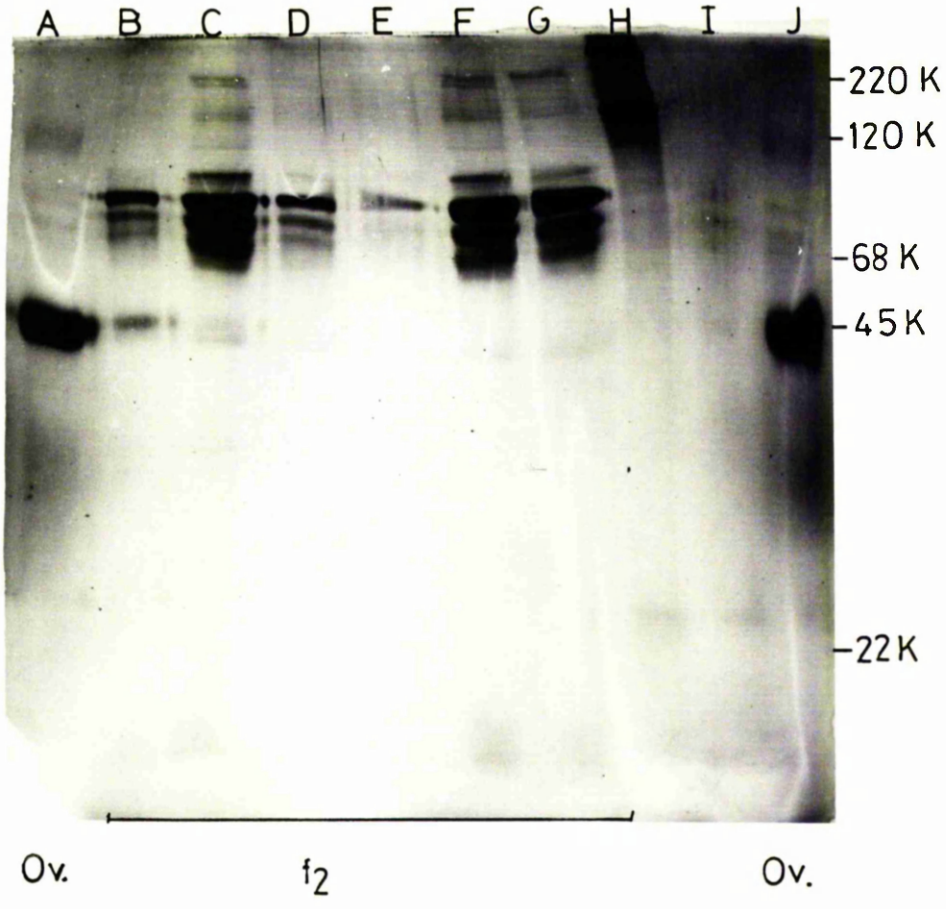
40µl C3H " " " (" E)

20µl B10.A(3R) " " " (" F)

30µl B10.A " " " (" G)

(Track H is not relevant to the work presented in this chapter and contains a sample obtained from C3H cell membrane preparations.)

This gel shows that f2 condtioned media contain a number of proteins around 60 - 100 K Daltons. These were not identified further.


A
B
C
D
E
F
G
H
I
J
220 K
120 K
68 K
45 K
22 K
Ov.
f2
Ov.

The conditioned media used above were obtained from cultures of spleen cells or PEC's which had been growing for 72 hours at 37°C. It could be argued that at some time after 72 hours, culture medium may have had a different effect on cell counts and spread cell area, or that those H-2K and I-A syngeneic conditioned media would also affect cell counts and area if taken from cultures which had been established for a longer period. To investigate these possibilities, culture medium was taken from cells after 1 day, between 1 and 3 days, 3 and 7 days, and 7 and 11 days, and each was examined separately for its effects on cell counts and spreading on three types of PEC's. The results (Table 20) showed that those culture media which had failed to have any effect on cell counts and spreading when removed from cultures after 72 hours, continued to show a lack of effect even when taken from cultures after 7 to 11 days.

Table 20.

Effects of f2 spleen and PEC conditioned medium on counts per 0.29 mm^2 and spread area (ppu) of PEC's do not change when they are obtained over varying time courses.

(Cultures started on Day 0). Results depicted in figs. 11a, 11b.

B10.HTT PEC's.

Cond. Med	Haplotype	Time of cond. day - day	Mean count	Mean Area
Uncond.	-	- -	65 (13)	234 (27)
B10.HTT	sssskkk?d	0 - 3	66 (15)	241 (28)
"	"	3 - 7	61 (16)	235 (18)
B10.S(7R)	sssssssd	0 - 3	64 (10)	229 (16)
"	"	3 - 7	65 (13)	235 (34)

B10.BR PEC's.

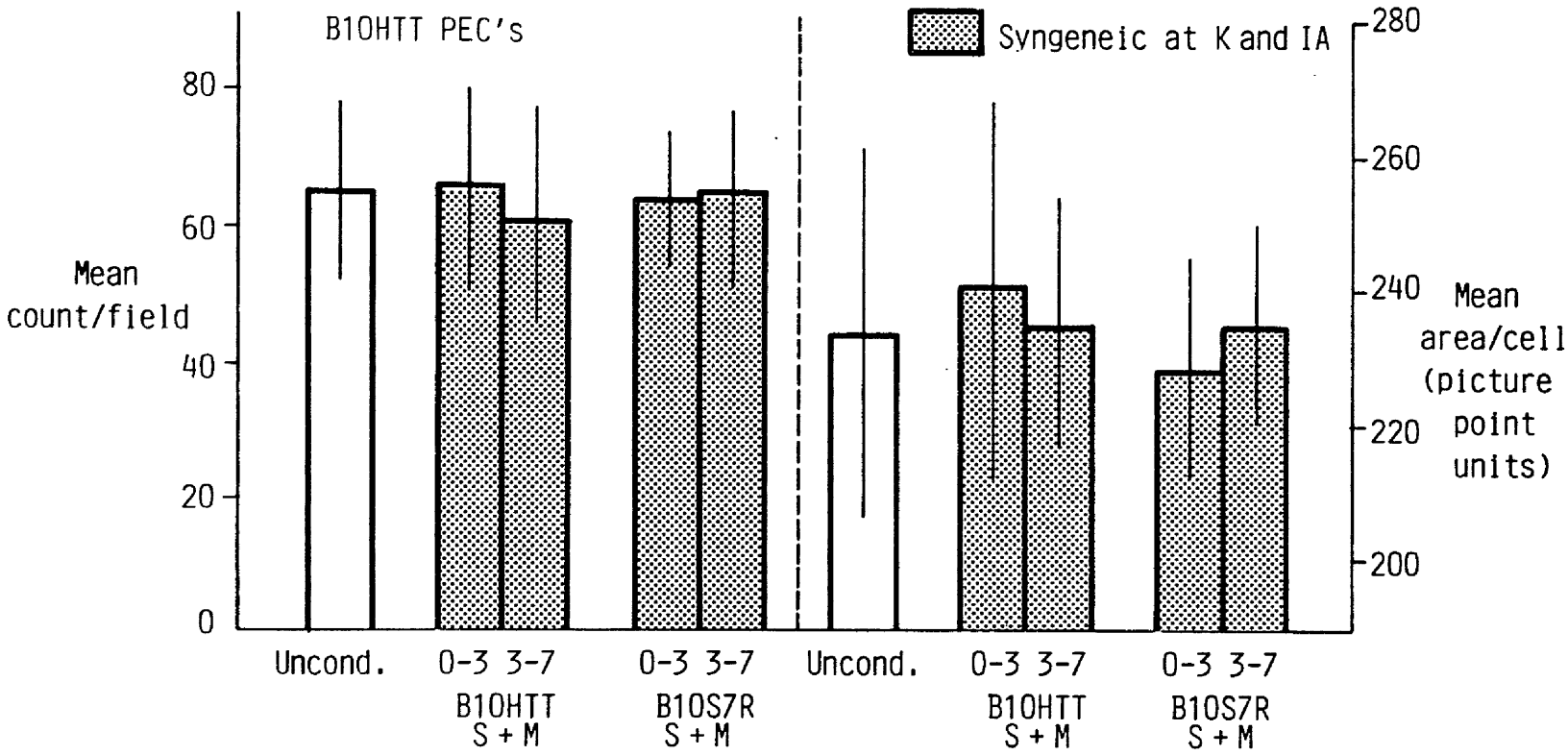
Uncond.	-	-	-	16 (3)	136 (13)
B10.A(4R)	(m)	kkbbbbbbb	0 - 1	15 (5)	191 (22)
"	"	"	1 - 3	22 (5)	187 (24)
"	"	"	3 - 7	26 (4)	184 (25)
"	"	"	7 - 11	23 (8)	181 (27)
"	(s)	"	0 - 1	15 (7)	187 (29)
"	"	"	1 - 3	15 (3)	183 (22)
"	"	"	3 - 7	16 (6)	202 (21)
B10.BR	(m)	kkkkkkkkk	0 - 3	21 (5)	175 (25)
"	"	"	3 - 7	25 (8)	154 (24)
"	(s)	"	0 - 3	21 (5)	155 (28)
"	"	"	3 - 7	22 (6)	162 (15)

Table 20 (continued) B10.A(4R) PEC's.

Cond. Med.	Haplotype	Time of cond. day - day	Mean Count	Mean area
Uncond.	-	- -	10 (7)	174 (22)
B10.A(4R) (m)	kkbbbbbbb	0 - 1	15 (7)	183 (21)
" "	"	1 - 3	12 (3)	180 (26)
" "	"	3 - 7	11 (6)	169 (18)
" "	"	7 - 11	10 (2)	167 (30)
" (s)	"	0 - 1	10 (2)	155 (35)
" "	"	1 - 3	10 (5)	151 (23)
" "	"	3 - 7	10 (4)	164 (22)
B10.BR (m)	kkkkkkkkk	0 - 3	15 (7)	163 (24)
" "	"	3 - 7	13 (5)	176 (25)
" (s)	"	0 - 3	14 (7)	177 (24)
" "	"	3 - 7	9 (4)	171 (12)
[B10.BR x B10.A(4R)] F_1	PEC's.			
Uncond.	-	- -	24 (6)	162 (17)
B10.A(4R) (m)	kkbbbbbbb	0 - 1	18 (5)	183 (21)
" "	"	1 - 3	17 (4)	201 (23)
" "	"	3 - 7	18 (7)	204 (12)
" "	"	7 - 11	23 (6)	208 (26)
" (s)	"	0 - 1	23 (4)	206 (18)
" "	"	1 - 3	25 (9)	198 (25)
" "	"	3 - 7	26 (8)	182 (30)
B10.BR (m)	kkkkkkkkk	0 - 3	27 (7)	190 (21)
" "	"	3 - 7	25 (3)	188 (23)
" (s)	"	0 - 3	21 (10)	196 (29)
" "	"	3 - 7	26 (4)	198 (23)

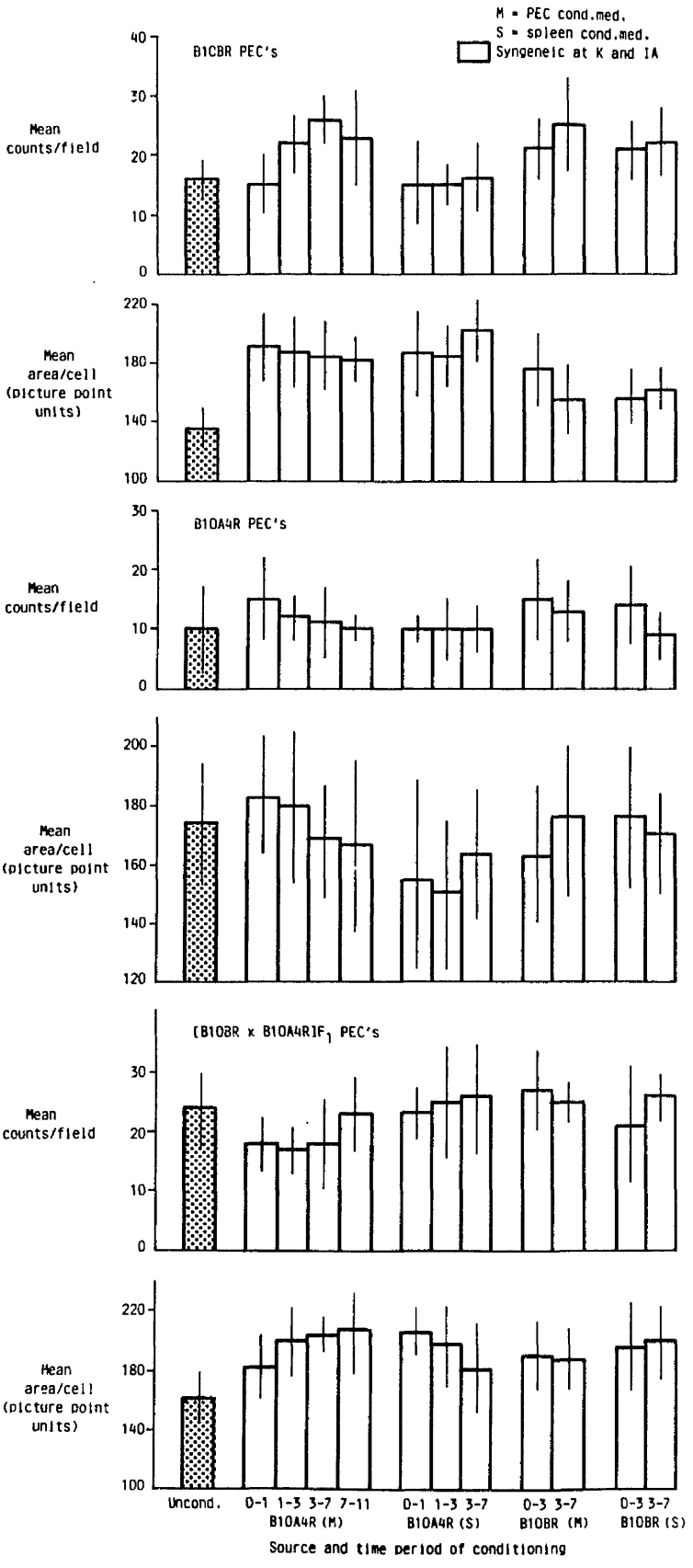
FIG. 11a.

Lack of effect on count and spread area of f_2 conditioned medium taken from strains syngeneic at K and IA is not dependent on "conditioning time"



S = spleen cond.medium
M = PEC cond.medium

FIG. 11b.


M = PEC cond.med.
S = spleen cond.med.
Syngeneic at K and IA
B1CBR PEC's
Mean counts/field
Mean area/cell (picture point units)
B10A4R PEC's
(B10BR x B10A4R1F1 PEC's
Uncond.
0-1 1-3 3-7 7-11
B10A4R (M)
0-1 1-3 3-7
B10A4R (S)
0-3 3-7
B10BR (M)
0-3 3-7
B10BR (S)
Source and time period of conditioning

The next experiment was designed to investigate the effects of mixing two conditioned media before adding to the PEC's in rotatory cultures. The conditioned media used were H-2 syngeneic and H-2 allogeneic, and comparisons were made between effects of each singly and effects of both types combined. The results are shown in Table 21 (fig. 12). Interestingly, the effects of the H-2 allogeneic conditioned medium was totally abolished when H-2 syngeneic conditioned medium was present.

The previous experiments have all shown that some factor present in conditioned medium obtained from 72 hour cultures of PEC's and / or spleen cells is capable of reacting with H-2K and I-A allogeneic cells to cause increased counts and spread cell area when the cells are added to glass substrates. The last two experiments were designed to determine whether H-2Iα products could be detected on the surfaces of H-2K and I-A syngeneic and allogeneic cells after incubation with H-2K and I-A allogeneic conditioned medium in rotatory cultures, followed by rigorous washing. Both the adherent sub - population and the total population of PEC's were examined. Complement - mediated cytotoxicity tests were performed using polyclonal anti Iα^{k} antisera and complement. These results (Tables 22 and 23) showed that allogeneic Ia fragments attach to PEC surfaces in an antigenically recognisable form. It should be noted that the inability to detect syngeneic fragments on the surfaces of PEC's may be due to a more transient interaction occuring in this case. Therefore, no conclusions can be drawn as to whether syngeneic Iα binds to PEC cell surfaces, except that the interaction differs from allogeneic Iα binding.

Table 21.

Effect of mixing different f2 spleen cell conditioned media on cell count per mm^2 and spread area (ppu) of PEC's.

Cond. Med.	Haplotype	Mean Count	Mean cell area.
B10 PEC's.			
Uncond.	-	30 (9)	199 (13)
B10	bbbbbbbbb	32 (9)	204 (25)
B10.BR	kkkkkkkkk	51 (8)	240 (17)
1:1 Mix	b/k	33 (7)	206 (25)
B10.BR PEC's.			
Uncond.	-	26 (12)	180 (20)
B10	bbbbbbbbb	58 (13)	283 (19)
B10.BR	kkkkkkkkk	33 (8)	224 (21)
1:1 Mix	b/k	34 (6)	218 (23)
[B10 x B10.BR] F1 PEC's.			
Uncond.	-	25 (13)	222 (16)
B10	bbbbbbbbb	28 (2)	234 (19)
B10.BR	kkkkkkkkk	21 (10)	227 (23)
1:1 Mix	b/k	23 (2)	212 (25)

These results are depicted in fig. 12.

FIG. 12.

Effect of mixing different f_2 spleen cell conditioned media on cell count and spread area of PEC's

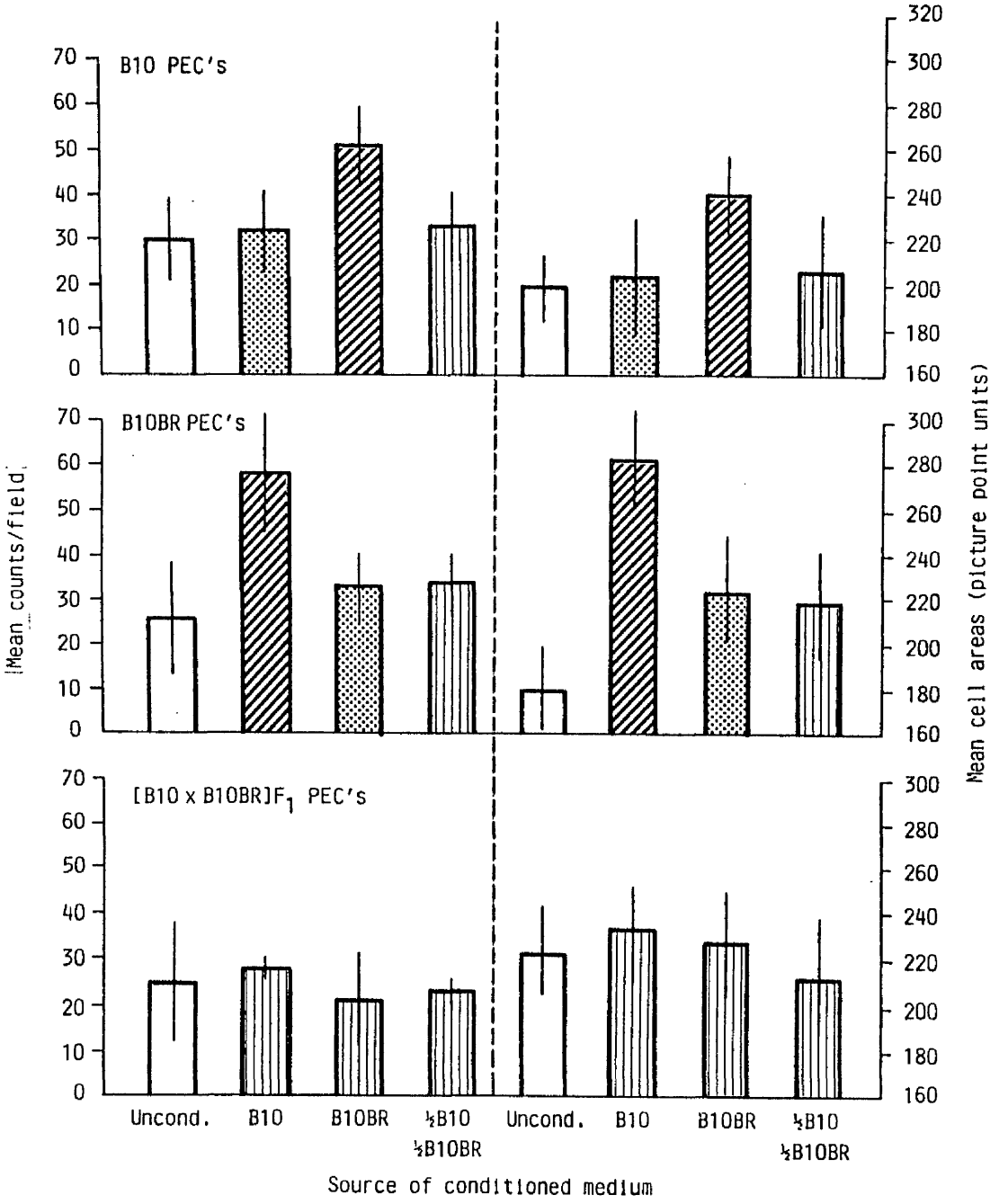


Table 22.

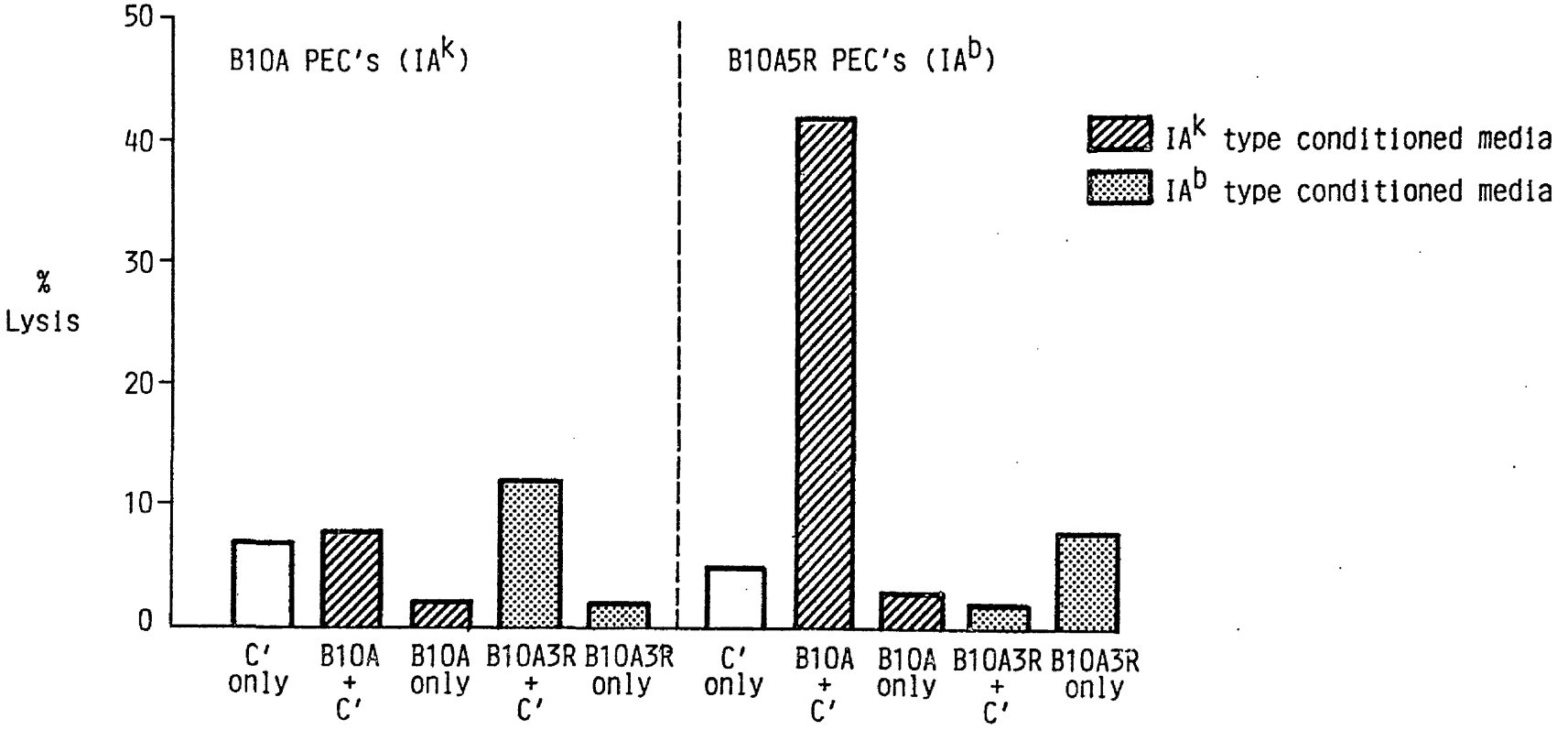
Detection of $I\alpha^k$ fragments by complement-mediated lysis on the surface of unactivated PEC's after incubation with fraction 2 of conditioned medium.

PEC's	Cond. Med.	I-A type.	C' added.	% Lysis
B10.A ($I\text{-}A^k$)	-	-	+	7
"	B10.A	k	+	8
"	"	k	-	2
"	B10.A(3R)	b	+	12
"	"	b	-	2
B10.A(5R) ($I\text{-}A^b$)	-	-	+	5
"	B10.A	k	+	42
"	"	k	-	3
"	B10.A(3R)	b	+	2
"	"	b	-	8

The above results were obtained from three experiments and percentages were calculated by counting at least 200 cells. These results are depicted in fig. 13.

FIG. 13.

Detection of Iα fragments by complement-mediated cytotoxicity on the surface of unactivated PEC's after incubation with f_2 conditioned medium



Standard deviations are not shown, but never exceeded ±5%.

Table 23.

Detection of $I\alpha^k$ fragments by complement - mediated cytotoxicity on the surface of unactivated adherent populations of PEC's after incubation with fraction 2 of conditioned medium.

B10.A PEC's (Adherent Population).

Cells Added.	Cond. Med.	I-A Type	% Lysis
4×10^5	None	-	11
"	B10.A	k	10
"	B10.A(3R)	b	12
"	C3H	k	8
3×10^5	None	-	11
"	B10.A	k	7
"	B10.A(3R)	b	10
"	C3H	k	7
2×10^5	None	-	16
"	B10.A	k	9
"	B10.A(3R)	b	14
"	C3H	k	13
1×10^5	None	-	12
"	B10.A	k	9
"	B10.A(3R)	b	13
"	C3H	k	12

Results were repeated and the same values (+ or - 2%) were obtained. Percentages were calculated by counting up to 500 cells. These results are shown in fig. 14.

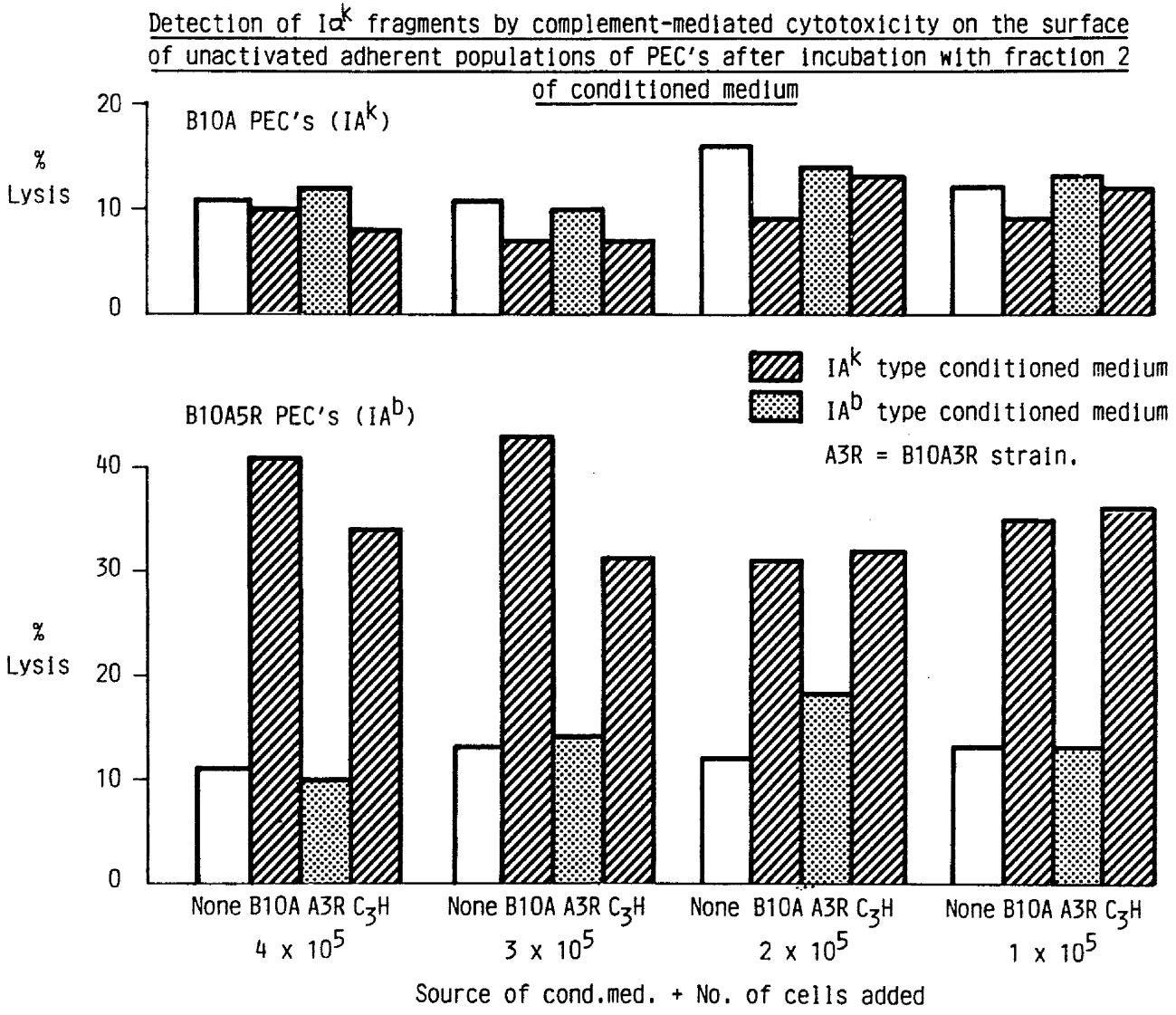
Table 23 continued.

B10.A(5R) PEC's (Adherent Population).

Cells Added.	Cond. Med.	I-A Type	% Lysis
4×10^5	None	-	11
"	B10.A	k	41
"	B10.A(3R)	b	10
"	C3H	k	34
3×10^5	None	-	13
"	B10.A	k	43
"	B10.A(3R)	b	14
"	C3H	k	31
2×10^5	None	-	12
"	B10.A	k	31
"	B10.A(3R)	b	18
"	C3H	k	32
1×10^5	None	-	13
"	B10.A	k	35
"	B10.A(3R)	b	13
"	C3H	k	36

Percentage lysis given in the above tables refers to lysis obtained using anti $I\text{-}A^k$ and complement in a complement - mediated cytotoxicity test as previously outlined. These results are shown in fig. 14.

FIG. 14.


Detection of Iα^k fragments by complement-mediated cytotoxicity on the surface of unactivated adherent populations of PEC's after incubation with fraction 2 of conditioned medium
B10A PEC's (IA^k)
% Lysis
20
10
0
IA^k type conditioned medium
IA^b type conditioned medium
A3R = B10A3R strain.
B10A5R PEC's (IA^b)
% Lysis
40
30
20
10
0
None B10A A3R C3H
4 x 10^5
None B10A A3R C3H
3 x 10^5
None B10A A3R C3H
2 x 10^5
None B10A A3R C3H
1 x 10^5
Source of cond.med. + No. of cells added

Conclusions

The differences seen between H-2K and I-A syngeneic and allogeneic effects on cell counts and spread area are significant at the p 0.05 level of the standard t Test.

It has previously been shown that spleen cells and thymocytes shed H-2 fragments into the medium (Curtis and Davies, 1981; Emerson et al, 1980). The results presented here support this work, and also show that PEC's similarly produce these factors. The H-2D fragments produced by T cells have been shown to interact with B cells of the same H-2 type, or with T cells which differ at H-2D, resulting in a reduced adhesiveness of the target cell to substrates (Curtis and Davies, 1981). The work presented in this chapter appears to be the first report of binding of allogeneic H-2 products to cell surfaces resulting in non - specific changes in adhesivity. H-2I (and possibly H-2K) fragments have been shown to bind to the surface of PEC'S and endothelial - like cells which are mismatched at these loci. Most interestingly, the bound fragments still retained their antigenicity, as they could be recognised by polyclonal anti Ia^k antibodies. This finding may be of importance in the mechanism controlling the immune response to cells bearing allogeneic H-2 products. As a result of the binding of H-2Ia allogeneic fragments, the cells show a non - specific alteration in adhesivity to glass substrates, manifested in increased mean cell counts and area. It would be interesting to determine whether T-I.M.F. reduces the adhesivity of B cells and H-2D allogeneic T cells by binding to their surface. It may be that I.M.F.'s control the adhesiveness of other cells by such a mechanism.

From Tables 15, 16, and 17 (figs 6, 7 and 8) there is some suggestion that fraction 2 of spleen cell conditioned medium may contain some factor not found in f2 PEC conditioned medium which decreases the number of cells adhering to glass substrates. The mean cell count results obtained for f2 spleen cell conditioned medium were consistently slightly lower than f2 PEC conditioned medium although areas are unaffected. Observations of the cultures from which f2 conditioned media were obtained revealed that spleen cell cultures showed an approximately 50% decrease in viability over a week in culture, whereas PEC's remained healthy for periods of up to 2 months. Consequently, the f2 spleen cell conditioned medium may have contained damaging enzymes etc. released from dead or dying cells, and these could be responsible for the decrease in cell counts seen when using this medium. It is also possible that spleen cells released additional factors which could affect cell counts and spread. The most interesting observation from Table 16 (fig. 7) is that when f2 conditioned medium was allogeneic at the K and I-A region, there was an increase in both counts and spread cell area on glass substrates. Non - H-2 mismatching alone of cells and f2 conditioned media is unlikely to have any effects on the spread area or counts (Tables 15 - 18, figs 6 - 9).

Generally, f2 conditioned media promoted greater spreading and gave an increased number of cells per field as compared to unconditioned controls. Reduction in cell spreading seen with some combinations of cells and f2 conditioned media was unlikely to be due to contact inhibition since this did not correlate with increased counts, and furthermore direct

observation of the preparations revealed that the cells were not in intimate contact with each other.

[B10.BR x B10.A(4R)] F_1 cells showed no increase in counts nor spreading when preincubated in either parental type f2 conditioned medium (Table 16, fig. 7). Where K, I-A, I-B, I-J, and D allogeneic combinations were used, an increase in counts and area was seen. This behaviour was synonymous with that found in the parental strains.

Table 18 (fig. 9) shows results in which endothelial - like cells from murine lung were treated with f2 spleen and PEC conditioned media obtained from various strains. Once again, if this was allogeneic at K, I-A, I-B and I-J increased counts and spread area were obtained. Thus, the effects are seen in cells other than PEC's. It would be interesting to examine the effects on many different cell types to determine if this phenomenon is seen.

Attempts to identify the factors responsible for the effects seen included the use of SDS-PAGE techniques. Both Kenacid Blue and silver stained gels of the three fractions of conditioned media were run. These showed (Plates 13, 14, 15) that the use of Amicon filters provided coarse separation according to molecular weight. New filters may have provided better separation; those used here had been extensively used for other preparations. Traces of proteins around 60 - 100 K Daltons were still present in the fractions which had been separated to exclude molecular weights of either >10K (fraction 1) or <10 and >35 K Daltons (fraction 2). Fraction 3 contained most of the >35 K Dalton proteins however, and large amounts were seen in the gels. From these results it is

difficult to determine which fraction might contain the H-2 fragments. The effects seen could be due to nanogram quantities which are undetectable on the gels by the method used. A further complication was encountered in that the conditioned media were adjusted to a total protein concentration of 1 mg/ml, and therefore the proportions of the constituent proteins were not standardised. The conditioning factor should therefore be isolated and set to a known concentration when comparing its effects on different cell lines. The standardisation used here was to grow a defined number of cells in medium for a set length of time, and has the disadvantage that this does not take into account the different growth or H-2 shedding rates of the strains used.

The effects of the three different fractions of conditioned medium on cell count and spread area were compared (Table 19, fig. 10). Fraction 1 had no significant effect on cell count or spread cell area, whether conditioned or unconditioned. Fractions 2 and 3 of the unconditioned medium tended to decrease the number and spread area of cells, whereas the same fractions of conditioned media tended to increase both counts and spread of B10.BR PEC's, particularly if taken from strains which are allogeneic at H-2K, I-A, I-B, and I-J.

The decrease in counts and area obtained with unconditioned f3 may be due to the presence of other molecules which either specifically or non - specifically decrease cell adhesiveness (see later discussion on lymphokines). For this reason, fraction 3 was not used in these experiments. However it should be noted that f2 and f3 had similar effects on cell count and spread when taken from the same strain (Table 19) and may both

contain the H-2 product or product which are responsible for these results. The SDS-PAGE technique confirmed the similarity of molecular weights found in each, although f3 contained a greater concentration of these.

The investigation was continued to find out whether those conditioned media which failed to give a response would do so if obtained from cultures which had been incubated for a greater period of time. From Table 20, (fig, 11), those conditioned media which failed to have any effect when obtained from 3 day cultures did not show any effects after up to 11 days culture.

Table 21 (fig. 12) shows the effects of mixing two different conditioned media. The results show that the effect of the allogeneic conditioned medium is abolished and the same result is obtained as for the syngeneic conditioned medium alone.

All results discussed here were analysed using the standard t test, and differences using f2 condtioned medium which was allogeneic at K and I-A were significant at $P < 0.05$ level.

From the above it is likely that an allogeneic cell product present in conditioned medium resulted in the increase in cell count and spread cell area. This was probably a product of the H-2 K or I-A region as shown by the use of congenic strains, Further evidence to support this was seen in the demonstration of allogeneic $I\alpha_k$ products on the surface of PEC's by complement - mediated cytotoxicity tests. Anti $I\alpha_k$ was added to PEC's which had been preincubated in conditioned medium, then those cells which had absorbed any $I\alpha_k$ were lysed with complement. Unactivated PEC's do not express $I\alpha_k$ products to any great extent.

Consequently, lysis would occur only of those cells which had bound I-A molecules from the conditioned medium. The first of these experiments was performed on the total population of PEC's to follow the experimental methods used in the rest of this series of experiments (see Table 22, fig. 13). 42% of the PEC's of the B10.A(5R) strain were lysed but only background levels of the B10.A PEC's. This indicated that PEC's would only absorb allogeneic $I\alpha$ molecules and not syngeneic $I\alpha$. These findings were supported by further experiments in which the adherent population of PEC's (approximately 90% macrophages) were reacted similarly with anti $I\alpha^k$ and complement (Table 23, fig. 14). Once again, only background lysis of the B10.A ($I\text{-}A^k$) strain was seen whereas between 31% and 43% of the B10.A(5R) ($I\text{-}A^b$) strain were lysed. This was not found to vary with the ratio of cell concentrations to conditioned medium used.

From these results it is not clear whether syngeneic fragments bind to the surface of the cells in a transitory interaction which is not detectable by these methods. The results suggest that the binding of allogeneic fragments promote adhesion and spreading of the 30 minute adherent population of PEC's. Whether this is due to I-A products alone or whether K locus products are also involved has not been resolved. The "allogeneic effect" seen here can be explained by considering the role of the macrophage in phagocytosis. After recognition of foreign proteins has occurred, it is conceivable that an increase in adhesion and spreading may follow to aid the phagocytotic activity. This will be discussed more fully elsewhere (see section 8.8).

CHAPTER 7. AGGREGATION OF ENDOTHELIAL - LIKE CELLS FROM STRAINS DIFFERING WITHIN H-2.

7.1. Aggregation Of Endothelial-Like Cells.

Introduction.

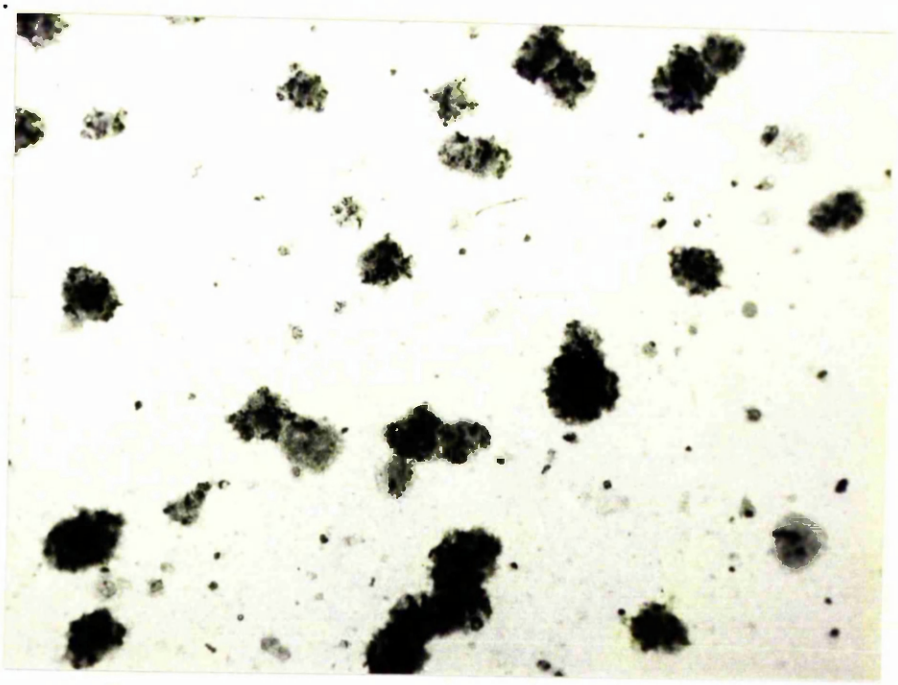
The aim of this investigation was to produce aggregates from cells of two different H-2 types, and to examine the final distribution of these to determine if sorting out by H-2 type would occur. The cell type used in these investigations was the endothelial-like fraction purified from murine lung.

Preliminary experiments showed that these cells would form aggregates of up to $1mm^3$ after 24 - 36 hours in a gyratory shaker. Before this time period, very small aggregates were obtained. Cells were collected on millepore filters after various time periods and were fixed and stained to determine aggregate size. Examples of these are shown in plate 16. After 7 hours, small aggregates of a few cells were obtained. Later these small aggregates tended to clump together to form larger aggregates (see plate 16b).

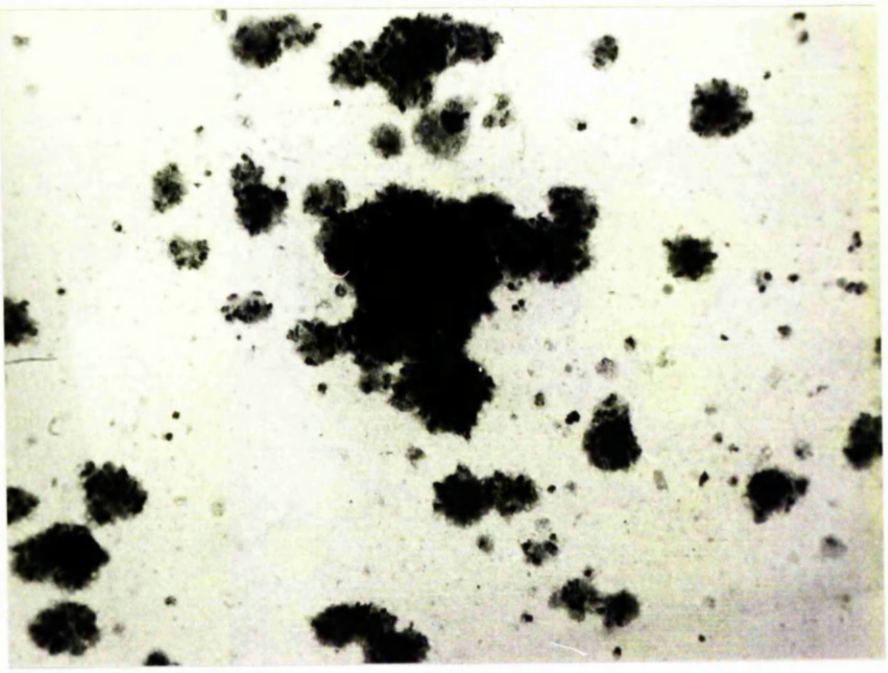
Plate 16.

Aggregation of endothelial - like cells from murine lung. A - 7 hour aggregates, B - 22 hour aggregates. These were collected on millepore filters, fixed in formol saline and stained with Haematoxylin and Eosin. In the 22 hour sample the smaller aggregates seem to be clumping together to form larger ones.

A.



B.



1mm

After establishing that aggregates could be produced, it was then essential to be able to recognise cells of different H-2 types within the aggregate. Therefore, one of the two strain types used was prelabelled with 5 µCi / ml tritiated thymidine, and scintillation counts (see page 57) showed that the cells were incorporating 0.5% of the tritiated thymidine added after 24 hours incubation. These labelled cells were envisaged in the final aggregate using autoradiographic techniques (see page 58). Percentage labelling was determined by aggregating tritiated thymidine - labelled cells only, then counting the proportions of labelled and unlabelled cells. It was important to determine the percentage labelling for recognition of cell type in the final aggregate.

All cells used were cultured by identical methods and in the same type of media (ie. Hams F10 + ITS + 3% HIFCS). In the following tables the number given in brackets is the number of aggregates examined. Each aggregate was produced in a different experiment, except where indicated. Experiments were conducted over a period of 3 months with no two experiments being performed on the same day. Furthermore, cells of each strain type were obtained from cultures of at least 90% pure endothelial - like cells immediately after selection. Ideally, cells of a particular H-2 type should have been obtained from different individuals of the same strain for each seperate experiment, but this was not done due to the time taken to establish each culture. These experiments are to be done in the near future.

Criterion For Recognition Of A "Labelled" Cell.

A cell was considered to be labelled if there were five or more silver grains over the nucleus, as the labelling intensity was high and the background low. Some workers have suggested a lower limit of four to five grains per cell (Baserga and Malamud, 1969), and others have referred to control autoradiographs of unlabelled tissues to determine the number of background grains over these nuclei (Stillstrom, 1963). However, the latter method assumes a uniform background count which is rarely achieved. I included unlabelled aggregate sections in all experiments and found that these showed less than five grains per nucleus in all cases.

Percentage labelling of cells in aggregates must be determined from aggregate sections. Furthermore, the thickness of the sections of the aggregate is critical since Tritium is a short pathlength beta emitter, and above certain thicknesses which are approximately equal to the pathlength, self - absorption occurs. In other words, the emissions are "quenched" by the source before reaching the emulsion. In the work presented here, 5µm sections were used. Rogers (1969) has published a table of autographic efficiency as a function of section thickness with a source uniformly labelled with Tritium. Tickle (1970) has discussed the problems associated with determining percentage labelling in some depth. Here, it is relevant to point out that 5µm sections provide a reasonable thickness of tissue section without encountering an excessive degree of quenching. To determine percentage labelling, cells of one H-2 type were cultures in Tritiated Thymidine as outlined in materials and methods, then were aggregated together

and 5μm sections taken for autoradiographic processing. This method has been used by Roth and Weston (1967) to determine percentage labelling. The results of percentage labelling in my experiments are shown in Table 24.

In experiments where one labelled cell type is mixed with an unlabelled cell type, problems are often encountered in exchange of label between cells. Trinkaus and Gross (1961) estimated exchange of label from labelled to unlabelled cells by reference to a second natural marker, ie. pigment granules in pigmented retinal epithelium. A similar experiment could be used here, in that the two different H-2 types of cells present could be envisaged by direct labelling of H-2 products. The problems arising with this technique are that very little of the cell surface is available for binding of anti - H-2 antibodies. However, some excellent results have been obtained in tissue sections from mouse chimaera's using alkaline phosphatase and immunoperoxidase double staining techniques (Ponder, personal communication). Further work should be done using these staining methods on aggregate sections.

The effect of labelling on cell behaviour was examined by mixing together "labelled" and "unlabelled" cells of the same H-2 type. These aggregates showed mixing of labelled and unlabelled cells, and it was therefore concluded that labelling did not affect cell behaviour in these experiments.

Table 24.

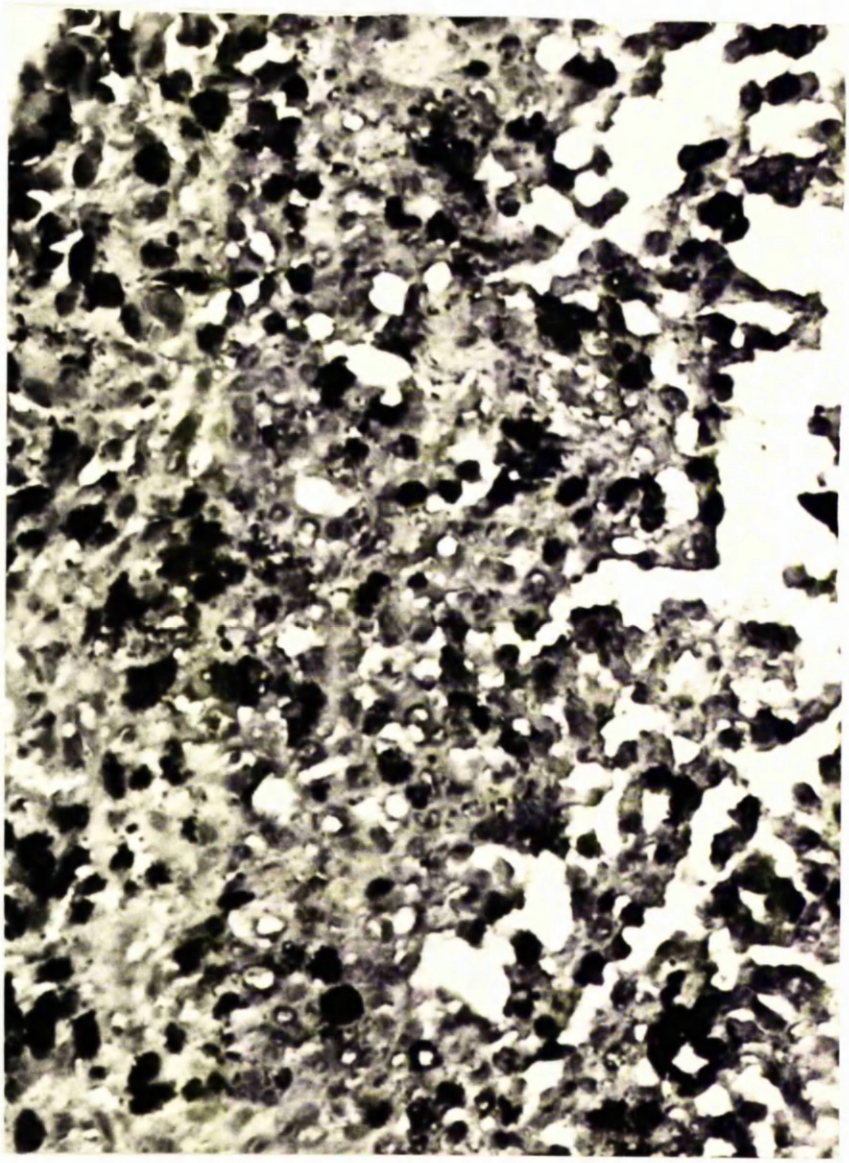
Percentage labelling of endothelial - like cells incubated with tritiated thymidine.

	% Labelled Cells			
Strain	Periphery of Agg.		Internal cells.	
B10.AKM	83	(3)	74	(3)
B10	76	(2)	77	(2)
B10.A(4R)	73	(2)	78	(2)

Percentages shown were obtained by counting at least 400 cells. The numbers in brackets show the numbers of aggregates examined of each strain type. Each aggregate was produced in a different experiment.

Plate 17.

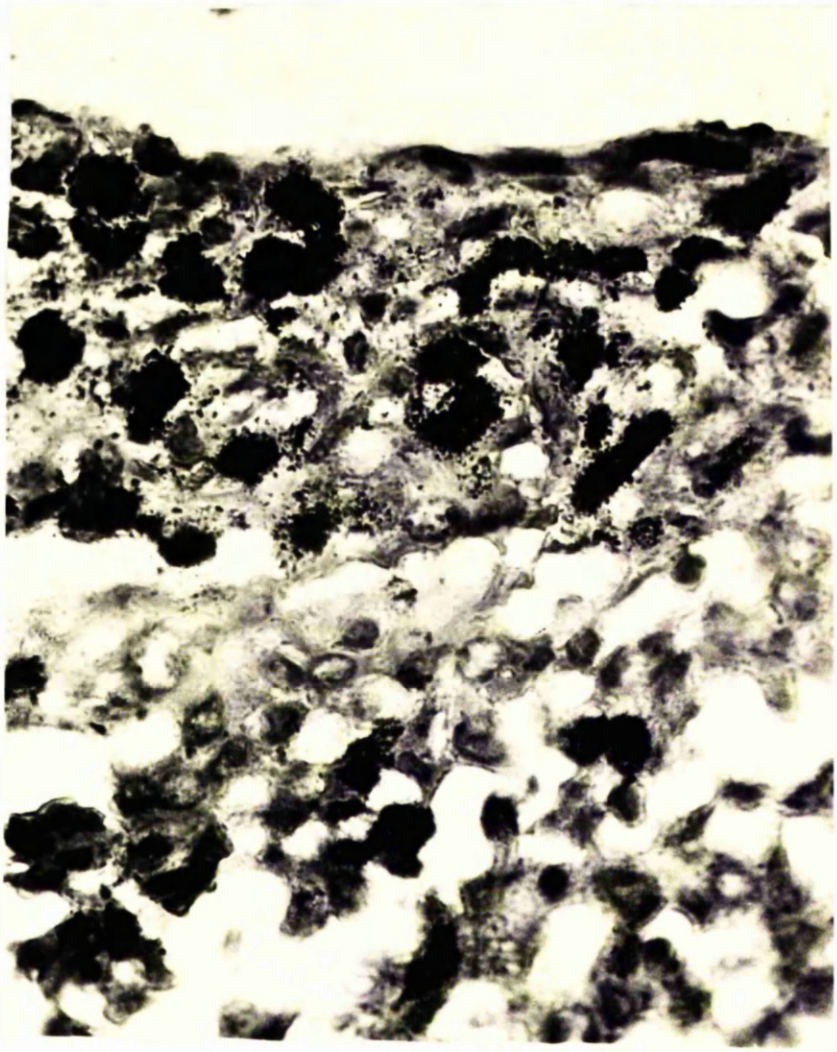
Autoradiograph of aggregate containing B10.A(4R) lung endothelial - like cells labelled with Tritiated Thymidine, mixed in a 2 : 1 ratio with B10 cells of the same tissue type. Strains are matched at H-2IB, IJ, IE/C, S, G, and D. No sorting out seen.



100 µm

Plate 18.

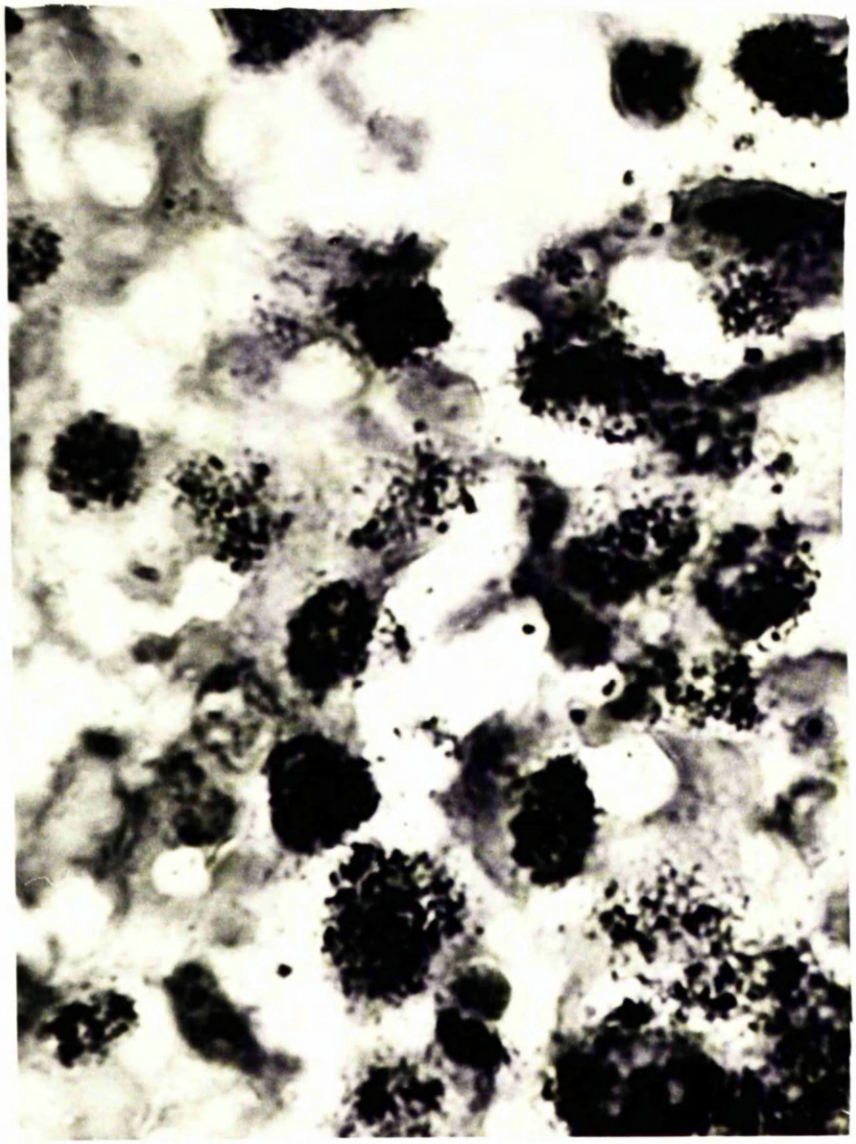
As Plate 17, but at higher magnification and showing the edge of the aggregate.



40 µm

Plate 19.

As in Plates 17 and 18, but showing mixture of labelled and unlabelled cells in the centre of the aggregate.



20 µm

The results (Table 24) show that 73 - 84% of cells are labelled. Some authors have maintained that 1,000 - 2,000 cells should be counted for a crude estimate of the percentage of labelled cells (Baserga and Malamud, 1969). However, some of the aggregates obtained here did not contain 1,000 cells and so a smaller sample size had to be used. Therefore, the results given in Table 25 were calculated by counting about 400 nuclei. There was very little difference between percentages of labelled cells on the periphery and inside the aggregate. Presumably, this was due to the cells being of one strain type only which had been purified to give approximately 90% endothelial - like cells.

Since the percentage labelling was 73 - 84%, for further experiments ratios of labelled to unlabelled cells of 1:1, 2:1, or 3:1 were used depending on the availability of labelled cells.

The next experiment was performed to determine whether endothelial - like cells mismatched at H-2K and I-A loci would sort out according to H-2 type in mixed aggregates. ^{3}H Thymidine - labelled B10.A(4R) cells were mixed in a 2 : 1 ratio with B10 cells, then the percentage of labelled cells inside and in the periphery of the aggregates was counted. The results are given in Table 25. No evidence for sorting out according to H-2 type was obtained by this method.

Another method was used to test for segregation according to type. This method was originally developed by Pielou (1962), and the theory will now be presented in detail.

Test For Segregation Of Cells According To Cell Type.

Consider two cell types A and B mixed together in an aggregate. If we take line transects across the aggregate recording the type of each cell in succession we can obtain a distribution of run lengths for each cell type. From Pielou (1962) the theoretical situation is as follows:

Let the probability of encountering individuals of A be a.

" " " " " " B " b.

The probability of obtaining an uninterrupted sequence of r individuals of A is $a^{r-1} b$.

The probability of obtaining an uninterrupted sequence of s individuals of B is $b^{s-1} a$.

So for both A and B the distribution of run lengths is geometric.

Considering the runs of A's,

let mean run length = m_A

Considering the runs of B's,

let mean run length = m_B

the estimator of b, $*b = 1 / m_A$

the estimator of a, $*a = 1 / m_B$

Let § be the proportion of A cells,

then (1 - §) is the proportion of B cells.

If the cells are randomly arranged

$1 / m_A + 1 / m_B = 1$

and $*b = 1 / m_A$ = proportion of B cells

and $*a = 1 / m_B$ = proportion of A cells,

so we can write

$$1 / m_A = (1 - §)$$

and $$1 / m_B = §$$

Therefore, in a random array we can check

$$1 / m_A + 1 / m_B = (1 - §) + § = 1$$

If the cells are segregated, the mean run lengths of A and B will be increased. $1 / m_A$ and $1 / m_B$ will be lowered from that expected from the proportions of A and B in the mixture. We can introduce the term ¶, to describe this lowering. ¶ = 1 when the cells are randomly arranged because in this case

$$1 / m_A = (1 - §) \quad \text{and} \quad 1 / m_B = §.$$

Introducing ¶

$$1 / m_A = ¶ (1 - §) \qquad 1 / m_B = ¶§$$

so the sum of $1 / m_A$ and $1 / m_B$ expressed in terms of ¶ is

$$1 / m_A + 1 / m_B = ¶$$ (¶ = 1 when cells are randomly arranged)

Pielou (1962) is interested in the value of

$$1 - (1 / m_A + 1 / m_B)$$

to detect segregation. If the cells are randomly arranged

$1 / m_A + 1 / m_B = 1$, and the difference is 0. When the cells are segregated, $1 / m_A + 1 / m_B$ tends towards 0 and the difference tends towards 1. If the number of observations of runs of A (n_A)

is sufficiently high, *b will have an approximately normal distribution. The variance of this distribution can be given as

$$S_b^2 = 1 / n_A \cdot \frac{m_A - 1}{3 \, m_A}$$

and similarly for the variance of a the same equation is used with B substituted for A above. If A and B are unsegregated it follows that with a 95% probability

$$1 / m_A + 1 / m_B = *b + *a$$

$$= 1 +/- 1.96 \times \sqrt{S_a^2 + S_b^2}$$

since the run lengths are independent of each other. We can test whether $1 / m_A + 1 / m_B$ is significantly less than unity and thus if the cells are segregated. We have the following observations;

m_L = mean run length of labelled cells

m_U = mean run length of unlabelled cells.

From these observations ¶ has been calculated and compared with 1 +/- 2 standard deviations, ignoring corrections for percentage labelling. The problems of assessing the degree of segregation in terms of the cell types, rather than the "labelled" and "unlabelled" cells is rather complicated as "unlabelled" runs will be heterogeneous with respect to cell type.

Transects.

The aggregates were all serial sectioned as previously outlined. That section which showed the largest diameter of the aggregate was used for the line transect. An eye piece graticule was used to obtain a line along which cells were scored according

to whether they were labelled or unlabelled at x1000 magnification. With the larger aggregates the field was moved so that the end cell was brought to the beginning of the line for the next field. This was repeated until the aggregate had been traversed.

This method was not used on all aggregates formed. This was because some had become torn during processing, or the autoradiography showed inconsistency across the section. In one combination, namely that of B10.S(7R) and B10.AKM, this method was not used since the aggregate contained a single layer of labelled cells around an unlabelled mass of cells in the centre of the aggregate. It was felt that the line transect method would afford very little advantage to the analysis of this mixture.

Table 25.

Distribution of aggregated cells mismatched at H-2K and -I-A.

Strains Used			% Labelled Cells.			
Labelled		Unlabelled	Periphery		Internal.	
B10.A(4R)	2:1	B10	53	(2)	56	(2)

Photographs of the aggregates are shown in Plates 17, 18 and 19. The above aggregates were all produced in different experiments. One aggregate was counted from each experiment. Haplotypes of each strain are as follows; B10.A(4R) is kkbbbbbbb and B10 is bbbbbbbbb.

Reciprocal labelling experiments were performed using B10.BR and B10.AKM cells in differing proportions. The results are shown in Table 26. It may be significant that when B10.AKM cells were labelled, more of these tended to appear in the periphery. However, further experiments are required in order to statistically confirm this finding, as the increase seen was not very marked. The recovery of cells added was rather low when a ratio of 7 : 3 labelled to unlabelled cells was used. This may have been due to defective handling resulting in a high cell mortality rate during the aggregation period.

The results of the transect method are given at the end of Table 26. Since $1 / m_L + 1 / m_U = 1$ and the difference is 0, there is no evidence for sorting out according to H-2 type in these aggregates.

Table 26.

Distribution of aggregated cells mismatched at H-2D only.

Strains			% Labelled Cells	
Labelled		Unlabelled	Periphery	Internal
B10.AKM	6:4	B10.BR	65	51
B10.BR	6:4	B10.AKM	55	60
B10.BR	1:1	B10.AKM	49	57
B10.BR	7:3	B10.AKM	54	50

Haplotypes of the above strains were as follows;

B10.BR kkkkkkkkk

B10.AKM kkkkkkkkq

Each of the above results was obtained from one experiment only. In each case, 2 aggregates were counted of each type and percentages were calculated as for one aggregate, since aggregates produced were small. This was probably a result of technical handling, since these combinations were performed early on in this series, and later aggregates were all much larger perhaps reflecting improvements in techniques. The results of the line transect through one of the 7 : 3 mixed aggregates indicate a random arrangement of cell types, as follows:

$*a = 0.5 \qquad *b = 0.5$

$m_L = 2 \qquad m_U = 2$

$\S = 0.5 \qquad \P = 1$

$1 - \P = 0$

$$\sqrt{S_L^2 + S_U^2} = v = 0.08331$$

$1 +/- 1.96v = 1 +/- 0.163328$

Aggregated Cells Mismatched At H-2K,-IA,-IB,-IC,-S,-G, and -D.

The strain types used were tritium - labelled B10.S(9R) and unlabelled B10.AKM. Only 30% of the peripheral cells were labelled, whereas between 44% and 71% of internal cells were labelled. This needs clarifying through further experiments. These results were obtained from one aggregate only, and line trasects were not taken on sections of this aggregate as it was very small.

Aggregated Cells Mismatched Throughout H-2.

Strain combinations used were tritium - labelled B10.S(7R) and unlabelled B10.AKM. These gave an interesting result whereby 98% of the cells on the periphery were labelled. In this combination, B10.S(7R) cells sorted out externally to B10.AKM. This needs to be confirmed by repeating the experiment and by reciprocal labelling of both types of cells in different aggregating cultures. Photographs of the aggregates are shown in Plates 20 and 21.

Another mixture of two cell types which differed throughout H-2 was aggregated. These were Tritium - labelled B10.BR and unlabelled B10.S(7R) mixed together in a 1 : 1 ratio. Here however, no evidence was obtained for sorting out according to H-2 type since 50% of the peripheral cells and 51% of the internal cells were labelled. Analysis of the aggregates using the transect method supported the above observations in that there was no evidence for segregation of cells according to H-2 type. The following values were obtained (see overleaf).

Transect Analysis Of Aggregate Containing Tritium Labelled B10.BR

And Unlabelled B10.S(7R) Cells (1:1 Ratio).

Total L	= 36	Total U	= 36
*a	= 0.5	*b	= 0.5
m_L	= 2	m_U	= 2
§	= 0.5	¶	= 1

1 - ¶ = 0

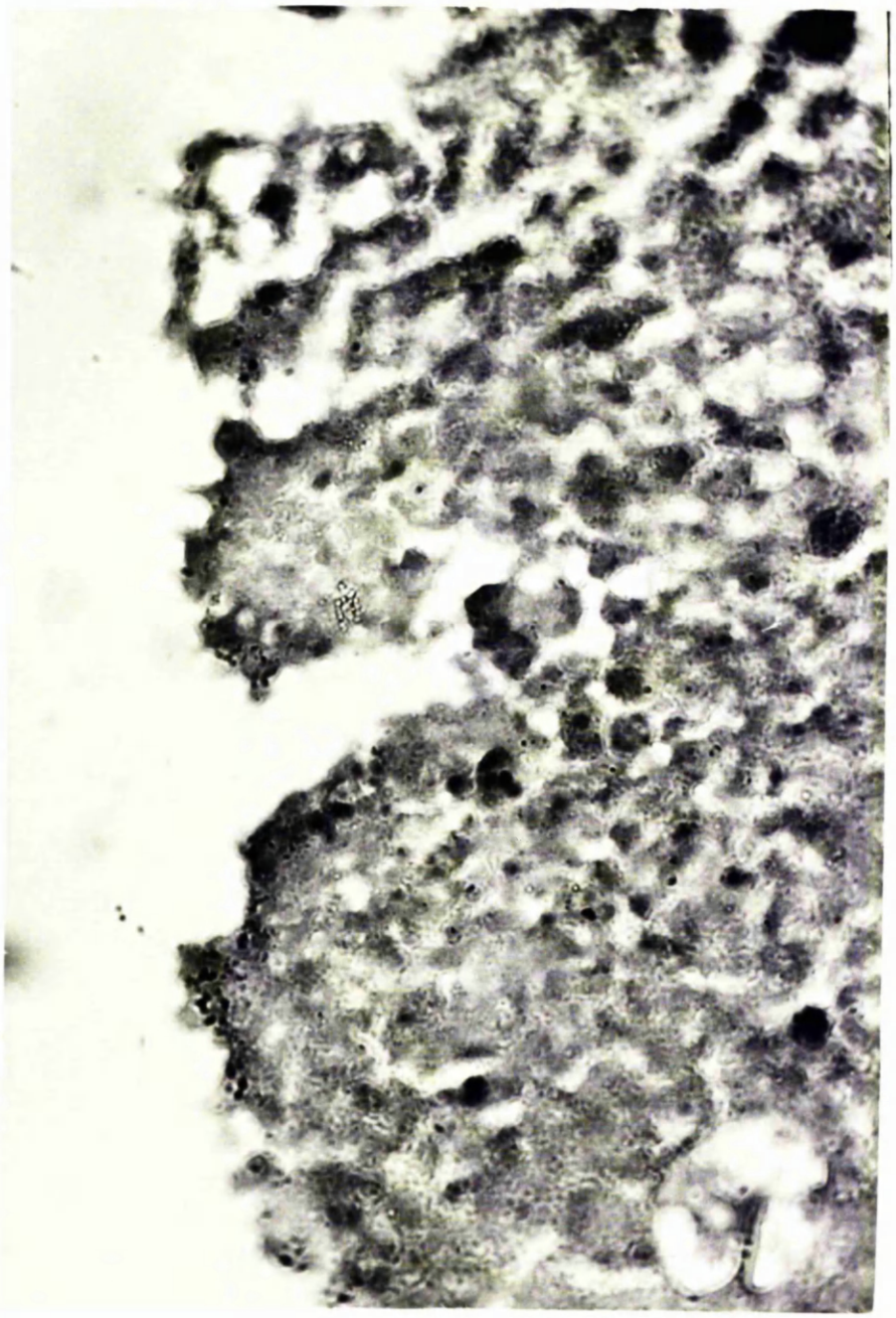
$$\sqrt{S_L^2 + S_U^2} = v = 1 +/- 0.163328$$

1 + or - 1.96 v = 1 +/- 0.163328

Referring to the theory presented on pages 154 - 156, there is no evidence for segregation of cell acording to H-2 type in this aggregate.

Plate 20.

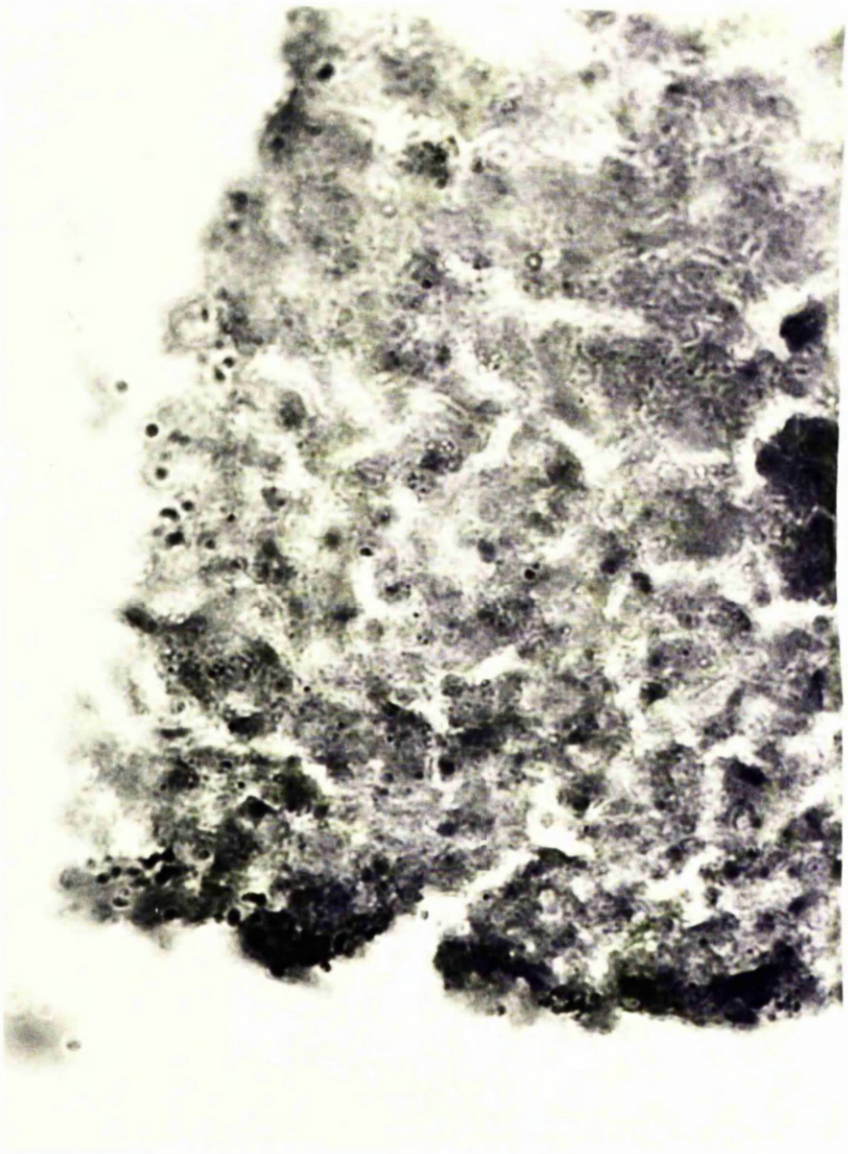
Autoradiograph of an aggregate containing B10.S(7R) lung endothelial - like cells labelled with Tritiated Thymidine mixed with an equal number of unlabelled B10.AKM cells. Strains are mismatched throughout H-2. Evidence for sorting out is seen in the arrangement of labelled cells around the periphery of the aggregate.



— 40 µm

Plate 21.

As in Plate 20, autoradiograph showing another view of the peripheral layer of Tritium - labelled B10.S(7R) cells surrounding unlabelled B10.AKM cells.



40μm

Effect Of H-2 Type On Aggregate Diameter.

The experiments in this chapter have been concerned with the possibility that cells in mixed aggregates may sort out according to H-2 type. This phenomenon, if it occurs at all, may operate by a different mechanism to that controlling aggregate formation between H-2 disparate cells. McClay and Gooding (1978) have previously investigated the ability of cells of different H-2 type to adhere to preformed aggregates. From their observations, they concluded that H-2 is not involved in the collection of cells to tissue fragments or preformed aggregates. I decided to measure the sizes of aggregates formed from mixtures of cells of either the same or different H-2 type to determine whether H-2 disparity affected the ability of cells to form aggregates. The greatest diameters of sections of each of the aggregates were compared. The results are given in Table 27. The variation within an H-2 type was quite large, and therefore comparisons between H-2 types, and between mixtures of two H-2 types, were not statistically possible. However, the results did imply that there was no marked effect of H-2 type on aggregate diameter. Thus, H-2 disparity does not seem to affect the ability of cells to form aggregates.

Table 27.

Effect of H-2 type on aggregate diameter.

Cells Aggregated	Greatest Diameter (μm)	No of cells in periphery
^{3}H B10.S(7R) + B10.S(7R)	300μm	28
	300μm	30
^{3}H B10.BR + B10.AKM	700μm	83
	100μm	15
	900μm	ND
^{3}H B10.S(7R) + B10.AKM	450μm	ND
^{3}H B10 Alone	130μm	24
3H B10.A(4R) Alone	80μm	33
	135μm	40
	180μm	66
^{3}H B10.BR	140μm	28

(ND = Cells not counted due to incomplete periphery.)

Conclusions.

In the aggregation experiment where B10.S(7R) cells appeared to sort out from B10.AKM cells it might be said that the sorting out seen is due to tissue type sorting out and not to H-2 type sorting out. However, if this were the case, it is unclear why B10.S(7R) cells failed to sort out internally to B10.BR in another aggregate, unless both these two types were identical and B10.AKM was different to both. A further complication of this theory is encountered in that B10.BR cells failed to segregate from B10.AKM cells which would have been expected from the above reasoning. This is presently under investigation using the latin square technique.

The ability of cells to form aggregates is probably H-2 independent as implied from Table 27, since aggregate diameter is not markedly affected by the H-2 types of the cells within it. These results do however require further statistical anlysis when more data is obtained.

The investigations reported above suggest that sorting out according to H-2 type may occur in mixed aggregates of endothelium as seen in the B10.S(7R) / B10.AKM combination above. The failure of B10.BR to sort out from B10.S(7R) or from B10.AKM may lend support to this conclusion. B10.BR and B10.AKM differ only at the D locus and both strains are allogeneic to B10.S(7R) at H-2D. It is possible that sorting out is not under the influence of the alloantigenic determinant on the H-2D products, though this remains to be investigated by the use of co - isogenic strains. Further work must therefore be done to determine if this is a real phenomenon.

CHAPTER 8. DISCUSSION.

DISCUSSION.

A summary of the main findings is given below. These will be discussed separately in detail later. Two different cell types were used in this work and some discussion on these is also given. Critical examinations of the methods and results are given in each section, together with possible interpretations of the findings. Conclusions are drawn separately at the end of each section, together with suggestions for further work. A more general review is then presented of the questions that remain to be answered on the role of H-2 products in cellular interactions. This will include a brief discussion of the work of others relevant to the findings presented here.

8.1. Summary Of Results.

The main conclusions which can be drawn from the previous chapters are as follows;

Cells purified from murine lung explant cultures were probably of an endothelial lineage.

Endothelial - like cells show heterogeneity of expression of Class 1 products as detected by monoclonal antibodies in immunofluorescent and complement - mediated cytotoxicity tests.

Peritoneal exudate cells adhere differentially to syngeneic and allogeneic H-2D fragment - coated substrates, as measured by differences in mean cell count and mean spread cell area. These findings support the work of Curtis (1983b), where endothelial - like cells from murine lung were shown to adhere

differentially to H-2K and -D coated substrates.

Medium taken from actively - growing cultures of spleen cells and / or PEC's can affect the adhesivity of PEC's and endothelial - like cells to glass substrates when allogeneic at H-2K and / or Iα . This is detected as an increase in spread cell area and cell counts which are significant at the P = 0.05% level of the t Test. Furthermore, using complement - mediated cytotoxicty tests, H-2Iα molecules were found to bind to allogeneic PEC cell surfaces in an antigenically - recognisable form. The binding of Iα fragments to syngeneic cell surfaces was not detected by this technique.

When endothelial - like cells of different H-2 type are mixed together in aggregating systems, they may sort out so that those of a type surround the other in a manner reminiscent of the tissue - type sorting out described by Steinberg (1964).

These results will be discussed at some length later in this text. Before this however, some consideration is given to the cell types used in the above-mentioned work.

8.2. Endothelial-Like Cells

Those cells purified from lung explant cultures on the basis of morphological criteria possessed many endothelial - like properties, as described in chapter 3. Transmission electron microscopy (TEM) sections of the endothelial - like fraction showed that coated vesicles were present in the cells and these have previously been described by others in pulmonary endothelium (Ryan and Ryan, 1977; Smith and Ryan, 1971; Barnhart and Baechler, 1978). Like other endothelia, the murine lung endothelial - like

cells showed a lack of characteristic organelles (Ryan and Ryan, 1977). Weibel Palade bodies (Weibel and Palade 1964) are an exception to this, but are not always present in endothelia. They are found particularly in young and foetal endothelial tissues of vertebrates, but are not particularly prominent in adult pulmonary endothelium (Ryan and Ryan, 1977). Plate 8b shows endothelial - like cells in TEM sections which contain organelles resembling the membrane bound packets of tubular material, 0.1μm x 3.0μm in size, described by Weibel and Palade (1964). The TEM results therefore suggest that the cells were probably endothelium. Other characteristics were sought to support this conclusion. These results were encouraging in their support. For instance, Factor VIII antigen was found to be present in the cells as detected by indirect immunofluorescent staining techniques. Some authors have previously described the Factor VIII staining as punctate, (Folkman, Haudenschild and Zetter, 1979) but this was not seen here. Explanations for this discrepancy may be found in the slightly variable properties of endothelial cells throughout the body. It is possible that the Factor VIII antigen is either incompletely synthesised or is rapidly degraded in these cells which may result in their diffuse staining. Angiotensin converting enzyme was shown to be present at high activity in these cells. This feature has been previously reported in pulmonary endothelium (for instance, see Ryan et al, 1978; Ryan and Ryan, 1977). The above-mentioned characteristics were supported by morphological and biochemical evidence and suggest strongly that the cells were of an endothelial lineage.

Some workers have reported that human endothelial cells

may be cultured to confluence only in the presence of endothelial cell growth supplement (ECGS) and 20% Foetal Bovine Serum on fibronectin - coated substrates. If ECGS and fibronectin are absent, cultures fail to achieve confluence beyond the first or second passage (Brattle, personal communication). The cells I used however showed no such dependancy on these supplements, achieving confluence in their absence. Folkman and Haudenschild (1980) have reported that human and bovine capillary endothelial cells require conditioned medium and grow best in tumour conditioned medium. In contrast, they found that endothelium from the aorta and umbilical vein do not require conditioned medium for growth. Capillary endothelia increase their rate of migration in a dose - dependant manner when increasing concentrations of tumour conditioned medium or tumour - derived growth factors are added. However, migration of aortic endothelium is not affected by tumour conditioned medium (Folkman and Haudenschild, 1980). In the endothelial - like cultures used in the work presented here, no conditioned medium was used, although the medium was supplemented with ITS and 3% HIFCS. It is possible that these lung capillary endothelial - like cells have different growth requirements to human and bovine endothelium, or perhaps this emphasises the varying properties of cells of the same tissue type obtained from different locations in the body. Further work on various types of endothelium is required to clarify these points.

It is well established that cells can change or lose their properties after extended periods in culture. Unfortunately, it was not possible to obtain purified endothelial - like cells from explant cultures without a number of passages. In order to

minimise the extent of these changes due to in vitro culturing purified endothelial - like cultures were used immediately after they had been selected out of mixed cultures. Surplus cultures were frozen to -70°C in 10% Dimethyl Sulphoxide in Hams F10 medium + 10% HIFCS and were restarted as required.

Ryan and Ryan (1977) pointed out that pulmonary endothelial cells occupy a unique position in the body. Lying between the blood and the lung parenchymal cells, they receive all the cardiac venous output and furthermore, the whole of the lungs venous effluent drains directly into the systemic circulation. As a consequence of this, it is possible that lung endothelium could exert humoral influences over specific functions of distant organs, although no evidence for this has been obtained to date. Pulmonary endothelium share many functions common to other endothelium. For instance, they are a barrier mechanism for the containment and regulation of blood elements; they act as a sieve for molecular transit and they are an integral and dynamic part of the physiological activities leading to enzyme induction and mitosis via the supportive functions of lipopolysaccharides and platelets (Barnhart and Baechler, 1978).

It is possible that the cells were not endothelium, even though the evidence strongly supports the contrary conclusion. Further experiments could be done to verify this, in order that attempts to repeat the work given here using this line could be standardised. Different cell lines could produce quite different results, therefore it is important to establish the identity of the cells used.

A discussion of the peritoneal exudate cells used will

be given here. This population contains a mixture of cells with very different properties. The most adherent population were selected for use in this project. These are likely to be predominantly macrophages.

8.3. Peritoneal Exudate Cells.

Peritoneal exudate cells were washed and either added to H-2D fragment coated substrates, or were mixed with conditioned medium in rotatory cultures on an Emscope. The cell counts and spread areas taken from each treatment would only be of that sub - population of peritoneal cells which adhere to clean glass or H-2D fragment - coated glass substrates within 30 minutes at 37°C. In one experiment, the adherent population was separated and tested for surface expression of I-A antigens after mixing with conditioned medium. These results will all be discussed later.

Cowing et al (1981) have reported that PEC's are composed of approximately 50% macrophages. Other cells present in their samples were presumably lymphocytes and a small number of other white blood cells. One of the major problems with these cells is their very marked heterogeneity. For instance, between 5 and 30% of peritoneal exudate macrophages bear Ia antigens (Cowing et al, 1981). The differences between these percentages is probably due to the transiency of Ia expression, which has been reported previously by Beller and Unanue (1981). Ia loss is seen after *in vitro* culturing of the cells and may in turn explain why grafts take better after culturing. *In vivo*, Ia antigens are also lost from the cell surface and a continuously renewing Ia-bearing population has been found. There does not appear to be a permanent

conversion of Ia-ve to Ia+ve phenotype and the two appear to be quite distinct populations. (For further details, see Beller and Unanue, 1981). Recently, a macrophage - like tumour cell line has been developed (Birmingham et al, 1982). This provides large numbers of homogeneous macrophages which can be used to study cell biological, biochemical and immunologial properties and functions of specific subpopulations.

Heterogeneity of cell populations and separation of a particular subpopulation forms a central problem in immunological research. Separations can be made according to one or more of a large number of criteria. Having acquired a subpopulation of PEC's for use in adhesion experiments, further problems are encountered in interpreting experiments where spread cell areas are measured. Indeed, there is no clear evidence to suggest that cell spreading is related to cell adhesivity to substrates. The problems inherent in any study of this relationship will now be considered.

8.4. Spread Area / Adhesivity Dichotomy.

As previously suggested, the relationship of spread cell area to cellular adhesiveness is unresolved at present. Two quite different explanations could be given for increased spread area on a given substrate. Firstly, consider a cell which has a finite population of 'adhesive receptors' on its surface. When an adhesive interaction occurs between this cell and a substrate, the cell spreads until all the 'adhesive receptors' are in contact with the molecules to which they can bind. Thus, spreading will occur to a greater extent on a low adhesive surface. If, however, cell adhesiveness is a result of non-specific molecular

interactions, analogous to the spreading of an oil droplet on a surface, then differences in cell spreading on a given substrate are directly related to the adhesiveness of the cell to that substrate. Further work is required to elucidate which of these two possibilites is correct. In this text, any suggestion that spreading is an indication of adhesiveness must therefore be regarded in the light of these reservations.

The ability of peritoneal exudate cells to bind to syngeneic and allogeneic H-2D fragment coated - substrates was examined. The results which are presented in chapter 5 will now be discussed in detail. Differential binding to these substrates was measured in terms of cell counts and spread area and conclusions can only be tentative until the questions raised above have been answered.

8.5. PEC Binding To H-2 Fragment Coated Substrates.

Attempts to study the involvement of H-2 in adhesive interactions between cells have generally employed cells which are mismatched at one or more H-2 loci (eg. Curtis and Renshaw, 1982; McClay and Gooding, 1978 etc.). Interpretation of the results of these studies is difficult, since one cannot eliminate any effects due to H-2 products blocking the action of non-H-2 cell surface components which are involved in adhesion. In chapter 5, an investigation of the involvement of H-2 in cellular interactions was done using a simplified model, ie; peritoneal exudate cells were added to syngeneic and allogeneic H-2D fragment coated substrates. Mean cell counts and spread areas were obtained from each after a 30 minute incubation period to determine whether

differential adhesion would occur. The results showed that PEC's adhere in reduced numbers to allogeneic H-2D fragment - coated substrates, but with increased spread area as compared to syngeneic H-2D fragment - coated substrates. This result is consistent with the first model given above (section 8.4) which envisages 'adhesive receptors' on the cell surface. Therefore, greater spread areas on allogeneic H-2D fragment - coated substrates may imply that these have a lower adhesivity as compared with syngeneic H-2D - coated substrates. This model does not clearly explain the increase in cell count which was seen using H-2K / I-A allogeneic conditioned medium (see 8.6.). The differential adhesion of PEC's to other H-2 products was not investigated and further work remains to be done to determine whether differential adhesion also occurs on H-2K or H-2I-A - coated substrates. However, the results suggest that H-2D products may play some role in cellular adhesive interactions, even if only in blocking cell substrate interaction sites. Increased spread areas but decreased counts on allogeneic substrate H-2D types might reflect the phagocytotic function of the adherent population of PEC's. This will be more fully discussed below.

The effects of conditioned media on the number of PEC's adhering and spreading on clean glass substrates was examined, and these will be discussed next.

8.6. Effects Of Preincubation Of PEC's In Conditioned Media.

Although the results discussed above suggest that H-2 products may be involved in adhesive interactions, it is unclear whether this is due to indirect effects. For instance, substrate

bound H-2 fragments could physically block the action of a non-specific substrate receptor on the cell surface. To determine whether H-2 interacts with the cell surface to produce a change in adhesive properties to substrates, experiments were designed in which H-2 fragments, which had been shed from actively- growing PEC's or spleen cells (Emerson et al, 1980) were allowed to interact with cells in rotatory cultures for 30 minutes. After this incubation, PEC's were rigorously washed to remove any unbound H-2 fragments, then were added to substrates. Any differences in adhesion of PEC's to clean glass substrates after this treatment was presumably a result of the interactions which had previously occured between them and the syngeneic or allogeneic H-2 fragments in the rotatory cultures on an Emscope. The conclusions of most of these experiments were discussed previously (chapter 6). It appears that when conditioned medium is allogeneic at K and / or I-A, there is an increase in both cell counts and in spread cell area on clean glass substrates. As a large number of combinations gave these results, it is likely that the effects are due to the differences in the antigenic determinant itself, rather than due to slight differences in the structure of different K or I-A alleles. Furthermore, these results are consistent with the findings discussed above, i.e. that cells spread out to a greater extent on H-2 allogeneic substrates, perhaps indicating a limited number of 'adhesive receptors' on the cell surface. However, this hypothesis fails to explain the increase in cell counts on H-2 allogeneic substrates. Further investigations, for instance, comparisons of the strength of the adhesion of cells to allogeneic and syngeneic substrates,

are required to test these theories.

As previously discussed in chapter 6, it is unclear whether syngeneic Iα fragments bind to the cell surface in a transitory interaction which is undetectable by these methods. If this does happen, there is no reason to assume that syngeneic and allogeneic fragments bind to the same cell surface molecule. However, the experiments where both syngeneic and allogeneic fragments were added simultaneously suggested that the effect of the allogeneic fragment was nullified by the presence of the syngeneic fragment and therefore the reaction promoted by the binding of the latter must take precedence over the two effects.

Many experiments have recently been done using conditioned culture media from lymphocytes or peritoneal exudate cells (or peritoneal exudate macrophages). For instance, T cells produce a factor which can replace T cells themselves in vivo (Taussig, 1974). It is relevant here to include a brief review of this work.

Interleukins are defined as factors prepared from leucocyte cultures which act on other leucocytes. Interleukin 1 is a macrophage / monocyte factor which does not induce B cell proliferation but does increase immunoglobulin production. Interleukin 2 is a T cell factor which acts on T helper cells resulting in an increased number of plaque forming cell assay (Duncan et al, 1982). Lymphokines are defined as lymphocyte factors which act on other lymphocytes. They have been obtained from human peripheral blood mononuclear lymphocytic cells after activation with antigen or mitogen. One of these is the lymphocyte mitogenic factor (LMF), a T cell product which can induce the

proliferation of B cells and immunoglobulin synthesis. This factor has a molecular weight of 27 - 35 K Daltons (Duncan et al, 1982). Salinas - Carmona et al (1982) have recently reported that a T cell factor of molecular weight 40 - 100 K Daltons was produced between 8 and 48 hours in cultures of peritoneal blood lymphocytes. This factor had suppressive effects on a number of immunological and non-immunological functions of a range of cells from lymphocytes to keratocytes. The factor was labile to treatment with low pH (pH 2) or temperatures of 70°C. Young (1982a and 1982b) has shown that conditioned media from lymphocytes, peritoneal cells, or peritoneal macrophages can stimulate the uptake of tritiated thymidine in lymphocytes. This work supported the findings of Petri and Fachetts (1981), whereby 2 factors were found to be present, one of which stimulated and the other inhibited DNA synthesis. Each factor was active under different circumstances, ie; depending on the mitogen used, whether lymphocytes were normal or transformed etc. It seems fairly likely that interleukins and lymphokines are synonymous since both are descriptive terms for the factors produced from white blood cells which affect other white blood cell types. The conditioned media described in this thesis is likely to contain similar factors, although the work in chapter 6 strongly suggests that I-A molecules are involved in the interactions seen here. Whether I-A products are responsible for the effects normally attributed to lymphokines or interleukin activity is unclear at present. However here Ia fragments were shown only to bind to allogeneic cells and could not be detected on syngeneic cells by this technique. Interleukins and lymphokines have been reported to show

H-2 restriction in some cases but not in others. Those factors which show antigenic specificity are often H-2 restricted, whereas non - specific factors are generally not H-2 restricted. Thus, the interleukin / lymphokine and allogeneic Iα absorption phenomena may not be comparable.

It is relevant here to discuss the implications of the adsorbtion of allogeneic Iα molecules onto the the surface of peritoneal exudate cells in more detail.

8.7. Adsorption Of Allogeneic Iα Fragments Onto PEC Surfaces.

The results presented in chapter 6 were of particular interest as they demonstrated that PEC's absorb allogeneic Iα fragments from conditioned medium onto the cell surface, resulting in an increased spread area and cell count. Thus, the presence of the absorbed allogeneic Iα fragments nonspecifically increases the spread area and number of cells on glass substrates. Whether the strength of adhesiveness is also altered by this interaction has not yet been tested here, but it would be of great interest to investigate this possibility further. Another interesting question concerns the mechanism which results in the increased spread area and cell counts after allogeneic Iα fragment binding (chapter 6). A number of possibilities could be put forward to explain this phenomenon. For instance, blocking cell - substrate adhesion sites due to steric effects could result in increased cell area if the cells had to expose more of their surface to the substrate to achieve the same stability of binding. This does not explain the increased number of cell counts however. Another possibility raised by Curtis (1983a) is that the binding of allogeneic H-2Iα

fragments acts as a triggering mechanism to nonspecifically increase cell - substrate binding and spread area. Syngeneic interactions would not result in any triggering according to this mechanism. This would be expected as syngeneic interactions would be constantly occuring in vivo between PEC's and shed H-2 fragments. Such an explanation accounts for both the increase in counts and also in spread cell area. However, the experiments in this section did not demonstrate clearly the role of K products, due to the lack of B10.MBR strains in the U.K. which allow analysis of K and I-A molecules separately. Further work is required to clarify this area.

The acquisition of allogeneic I-A molecules on cell surfaces has previously been described by Prud'Homme et al (1979), and by Delovitch et al (1979, 1982) when I-A molecules from irradiated host T cells were absorped onto donor graft-versus-host activated T cells. Also, T helper cells can absorb macrophage - derived syngeneic Ia antigens onto their surfaces (Paraskevas and Lee, 1979; Puri and Lonai, 1980; Yu et al, 1980; Nepom et al, 1981). In radiation - induced bone marrow - derived chimaeric mice, approximately 20% of the donor bone marrow - derived thymocytes bind host Ia antigens on their surface as they mature in the host thymic enviroment (Sharrow et al, 1980). However, this appears to be the first report of PEC absorption and expression of allogeneic but not syngeneic Iα molecules.

8.8. Effects Of Conditioned Medium On Endothelial-Like Cells.

Endothelial - like cells from murine lung also showed increased counts and spread area when incubated with H-2K and I-A

allogeneic conditioned medium. It would be of interest to determine whether these cells also absorb allogeneic Iα molecules, or whether the K fragment is of importance in this response. Pulmonary endothelial cells undergo a certain degree of pinocytotic activity, as demonstrated by the presence of coated vesicles in TEM sections. However, unlike macrophages, they tend to be stationary in vivo with a less well - defined immunological role. Consequently, the effects of the immune response gene products may be secondary to the effects of Class 1 (eg.H-2K) products. Class 1 products have previously been shown to be of importance in endothelial cell - lymphocyte adhesive interactions, (Curtis and Renshaw 1982) though no evidence has been obtained to date for a non - specific effect of allogeneic H-2K or D fragments. More work remains to be done to clarify this point.

Further evidence in support of the hypothesis which envisages a cell surface interaction mechanism by syngeneic and allogeneic fragments was obtained when two conditioned media were used separately then mixed. Here, the effects of the allogeneic fragments were abolished due to the presence of syngeneic fragments. Thus the syngeneic fragments allow normal reactivity whereas allogeneic interactions cause increased counts and spread.

F_1 cells showed similar effects to parental type cells, ie; allogeneic interactions resulted in increased counts and spread area. Semi - syngeneic conditioned media produced the same effects as syngeneic. This supports the experimental results using mixed syngeneic and allogeneic conditioned media discussed in the last paragraph.

8.9. Repeated Exposure To Antibody And Complement.

Klein (1975) has previously demonstrated that cells do not express as much H-2 antigenicity at the cell surface during the exponential phase of the growth cycle as compared to the stationary phase. Thus, a single exposure of a cell population containing cells in different stages of the growth cycle with specific antibody and complement would not be expected to lyse all the cells present. Experiments using B10.AKM cells showed that this was indeed the case. 98% of semi - confluent B10.AKM cells showed K^k expression as detected by immunofluorescence (Table 9) and yet the greatest population reduction after 1 treatment with antibody and complement was seen after 48 hours and indicated that only approximately 50% of the cells had been lysed (Table 4). Thus some of the cells which failed to react with antibody and complement may have been at the exponential phase of the growth cycle, although this is unlikely as it would have been detected by large increases in cell number in the control cultures over the incubation time. It is unclear at present why immunofluorescent techniques detected H-2 products on almost all the cells in the population when complement - mediated cytotoxicity did not. Predictions would have been that the two techniques would give the same results and one explanation is that the cells used were at different stages of confluency, although care was taken to use only semi - confluent cultures which were unlikely to be undergoing rapid growth. Confluent cultures could not be used in these experiments due to technical difficulties in performing accurate cell counts after treatment. Non-specific binding in the immunofluorescence experiments is unlikely to have occured as it

would have been detected in the controls. Taking the above conclusions further, repeated exposure at intervals out of phase with any time periodicity of the growth cycle would be expected finally to lyse all of a cell population expressing the allele against which the monoclonal used was directed. However, even after five consecutive treatments, some B10.BR and [B10.AQR x B10.A] F_1 cells remained which had not been lysed (Table 8).

One of the major problems with this technique is that after depleting the population with previous treatments, the survivors could go into the exponential phase and be less susceptible to treatment as a result of reduced H-2 expression. However, in some experiments the number of survivors remained constant over a period of three days yet susceptibility to treatment did not increase. Therefore, the stage of growth cannot be the only factor responsible for reduced sensitivity to treatment with antibody and complement.

Interestingly, B10.A cells were the most affected by these repeated exposures, suggesting a more homologous population with respect to susceptibility to treatment. However, the results using other strains implied that homology of K^k expression within a population was not always the case, as will now be discussed.

8.10. Heterogeneity Of Cell Surface Expression Of H-2K^k And D^k.

The results presented in chapter 4 indicate that homozygous pulmonary endothelial - like cells of some strain types are hetergeneous with respect to cell surface expression of H-2K^k and H-2D^k antigens as detected with monoclonal antibodies in complement cytotoxicity or indirect immunfluorescence tests. This phenomenon has previously been reported by others in spleen cell populations (Emerson et al, 1980; Oi et al, 1978) and may have been responsible for the partial rejection of F$_1$ skin grafts by parental rats reported by Anderson (1960). Results given in Table 3 show that F$_1$ heterozygotic cells are lysed to a lesser extent than parental homozygotes (ie. 45% of [B10.BR x B10.AKM] F$_1$'s (H-2D^k x H-2D^q) were lysed with anti D^k as compared to 69% of B10.BR cells). This could be a dosage effect, for instance F$_1$ cells may not have as many H-2 molecules of each parental type on their surfaces as parental cells although the efficiency of complement-mediated cytotoxicity tests does not support such an explanation. There may even be a variation in the number of each parental antigen present on the surface of F$_1$ cells of a homologous population. It is unlikely that allelic exclusion is occuring since F$_1$'s showed the same degree of susceptibility to repeated exposures with monoclonal antibody and complement as parental strains. It is unclear whether the heterogeneity of K^k expression within a homozygous population is a result of a variable number of H-2K^k products on the surface of an otherwise homogeneous population of cells, although the sensitivity of complement - mediated cytotoxicity tests is sufficient to detect

single antigenic structures on the cell surface. This would cast some doubt on this explanation. Furthermore, high standard deviations in experimental cultures as compared to controls reflected the patchiness of cell lysis, indicating that clonal depletion had occurred (Table 4). This suggests an inherited pattern of H-2 expression in daughter cells. Effects on H-2 phenotypic expression of diffusible substances from neighbouring cells could cause similar patchiness, but again this implies that at least 2 sub-populations are present. Clearly, a number of explanations could be put forward to explain these results. Some of these will now be briefly considered.

The first explanation concerns the genetic sequence of the H-2 antigens on each pair of chromatids. Cells of a homogeneous population may differ in primary structure. Robertson (1982) has put forward an explanation of how these differences may arise. Briefly, a small sequence of triplet codes may be transferred from one chromatid of a pair to the other. This gives rise to slightly different base sequences of the same protein and hence gene products could differ slightly. Since monoclonal antibodies are directed against one particular site on the protein, it is possible that these may be absent after sequence transfers have occured. This is consistent with the clonal depletion seen in the results.

Another explanation concerns post - translational events. $H\text{-}2K^k$ synthesis may be partially suppressed such that only some cells of a population are permitted or stimulated to express $H\text{-}2K^k$ antigens. Environmental signalling <u>in vivo</u> might occur via hormones etc which may be received and degraded by some but not

all of the endothelial cell population. However it is difficult to envisage how this could be transferred and maintained by cells of a clone *in vitro*, unless some kind of programming had occured *in vivo* before the lungs were removed. Rates of translation may vary in different cells, or there may be different rates of shedding of H-2 products from the cell surface.

Finally, H-2 products may be only partially 'unmasked' at the cell surface, or perhaps only partially synthesised. According to the above mentioned results on *in vitro* detection of K^k expression, the controlling factors of such a process must also be present in *in vitro* cultures after serial passaging. Evidence for such a system in embryonic cell types has previously been obtained using cell lines derived from 4 day mouse blastocysts (Ostrand - Rosenberg et al, 1977), but its operation in adult tissues remains speculative. Indeed, whether Class 1 products are expressed on embryonic tissues throughout development is still a matter of some controversy.

In conclusion, any of the above explanations could be operating to result in heterogeneity of H-2K^k expression within the population of endothelial - like cells. A cautionary note should be borne in mind in that the heterogeneity seen may be an artefact of *in vitro* passaging. It would be of interest to investigate whether this phenomenon was paralleled by any functional heterogeneity within the population. No experimental evidence has been obtained to date to answer this question. Furthermore, similar heterogeneity should be sought *in vivo* within homozygous individuals to determine whether this is simply an artefact of *in vitro* culturing techniques.

A more general observation was made during the above experiments, concerning the delayed effects of complement - mediated cytotoxicity on cell lysis. These will be discussed here.

8.11. Delayed Lysis Of Endothelial-Like Cells After Complement-Mediated Cytotoxicity.

Perhaps one of the most suprising observations obtained was the time interval seen between reaction with antibody and complement and the maximum decrease in viability. A video recording of endothelial - like cells after treatment revealed this delay and further observations where viable counts of B10.AKM cells were monitored over a period of 72 hours after treatment (Table 4) demonstrated that the greatest reduction in cell density was seen after 48 hours. In another experiment using B10.A cells, a delay of 96 hours gave the greatest reduction in density of viable cells (Table 5). This indicates that different strains may vary in the time taken to reveal cell damage. Delayed lysis may result from a disturbance of the ionic fluxes across the membrane which affect the internal biochemistry of the cell but do not cause immediate lysis. Thus, cells could undergo one or more rounds of division before this damage becomes apparent. It is possible that different strains show varying degrees of susceptibility to such damage, which may explain the variability with time after treatment before maximum reduction in viable cell density is seen between strains.

In Table 5, B10.A cells failed to recover to control levels as fast as in other experiments (Tables 7 and 8). Thus, differences in susceptibility to damage between strains may also

be coupled with variation within a strain, which could reflect slight differences in the culture conditions of each experiment. However, care was taken to ensure that conditions were kept standard as far as possible and it is therefore unlikely that such an explanation is applicable.

It would be interesting to investigate the possibility of a correlation between heterogeneity of expression as detected by complement - mediated cytotoxicity and the time delay before damage is manifested in terms of reduced viability. Such an approach may reveal more on dosage effects. Synchronised populations at the stationary stage of the growth cycle where cells are expressing a maximum amount of the appropriate allele could be purified on a fluorescence activated cell sorter to check that immunofluorescent detection correlated with complement cytotoxicity. In chapter 4, immunofluorescence results demonstrated that 59% of [B10.AQR x B10.A] F_1 cells expressed K^k as detected by 11.4.1 monoclonal antibody (Table 9) and this correlated with a failure to reduce the population by more than 50% using repeated exposure complement - mediated cytotocity tests (Table 8). Further experiments are required using other strains, and using purified populations before more conclusions can be drawn.

The above-mentioned heterogeneity may have consequences in the interpretation of experiments where H-2 fragments are used in suspension cultures or bound to substrates. Investigations involving H-2 fragments bound to substrates and H-2 fragments in suspension showed that differences in cell counts and spread area are seen depending on the H-2 types of the cells and the fragment

used. If heterogeneity of expression of H-2 products as detected by monoclonals reflects a heterogeneity in structure, the added fragments may have differing effects due to their differences in structure. H-2 fragments could be prepared from cloned populations before use in these experiments, although this would again lead to difficulties in interpretation, particularly if slight differences in the structure of the H-2 fragments of each clone led to slight differences in effects. Thus H-2 products from each different clone might give different results even though they were derived from one animal donor.

8.12. Differences In Trends Between Experimental Results.

One of the main differences in the results presented in this thesis was that PEC cells showed increased areas with decreased counts when adhering to H-2D allogeneic substrates as compared to syngeneic and yet if preincubated with conditioned medium no effect were seen due to H-2D allogeneity. Instead, effects were found to be due to H-2K and / or - I-A disparity, and then both counts and spread area were increased. It is possible that two unrelated mechanisms are involved in these interactions, since it is well established that Class 1 and Class 2 products are often involved in different interactions. It is also unclear whether the results are due to specific interactions, non-specific interactions, or simply a result of steric effects as previously discussed. Further work would include experiments whereby purified H-2K, - D, and - I-A fragments were added individually or simultaneously to PEC's to determine the involvement of each in PEC - substrate interactions. The finding that allogeneic H-2Iα

fragments bind to the surface of PEC's may have implications in vivo in graft - versus - host reactions. Phagocytosis may be promoted by any interaction which leads to an increase in spread cell area. Since the binding of an allogeneic Iα fragment leads to increased area and count on glass surfaces, Iα fragments are implicated in phagocytosis. This is not suprising if we consider the role of the macrophage, ie. the most prevalent cell type in PEC's, in antigenic presentation, which is itself an I-A - related phenomenon involving phagocytosis.

8.13. Aggregegation Of Endothelial-Like Cells.

The final set of results which will be discussed here are those in which aggregates were made of cells matched at various H-2 loci. These showed no sorting out behaviour. However, when cells were aggregated which differed completely throughout H-2, quite different results were obtained, as follows.

Experiments on the aggregation of endothelial - like cells from murine lung are still continuing. The results given in this thesis show that those combinations of cells tested which were mismatched at most but not all of the H-2 loci did not sort out into separate areas. However, one of the combinations which was totally mismatched at all H-2 loci showed sorting out in a typical 'sphere within a sphere' arrangement as previously described for the sorting out of tissue types (Steinberg 1964). This result has not been previously reported. Further work remains to be done to determine whether reciprocal labelling of the two types would produce the same arrangement (ie; B10.AKM cells inside, B10.S(7R) cells outside). The positional information which

determined the final arrangement of the two types with respect to each other is likely to be H-2 dependent, as this was the only difference between the two cell types. It is unlikely that the cells were separating out according to tissue type since purified cultures were used in which the contaminating cell type (fibroblasts) did not exceed 10% of the total population as determined by morphological criteria. Furthermore, B10.S(7R) and B10.BR both independently failed to sort out from B10.BR in other experiments. Investigations are continuing to verify that sorting out is not due to tissue - specific differences, using the latin square technique.

The following questions also remain unanswered: Does B10.AKM always segregate internally to cell types which are totally mismatched throughout H-2 and which particular loci are involved in this phenomenon ? Is sorting out according to H-2 type also seen in other cell types, eg. fibroblasts, or is it a property intrinsic to endothelial - like cells ? What effects would the addition of H-2 fragments before or during aggregation have on sorting out ? What is the time course of sorting out ? How long does it take for cells of a type to migrate to preferred sites in the aggregate ? If we mixed cells of two tissue types of known preferred final position, could this in any way be affected by also using different H-2 types which tended to segregate in the opposite way (ie. which is the strongest force, tissue type sorting out or H-2 type sorting out ?) These experiments may promote further interpretation of the previous work done by Steinberg (1964) which has not been followed up.

McClay and Gooding (1978) added two cell types (brain or

liver of either C3H or C57.Bl.10 strains) which had been labelled with ^{3}H or ^{14}C leucine to preformed aggregates of either C3H or C57.Bl.10. They reported that H-2 disparity had very little effect on the ability of either cell type to collect on the aggregates and concluded that there was no evidence of preferential adhesion of cells of the same H-2 type, although cells of the same tissue type did show preferential adhesion. In the work presented in chapter 7, H-2 disparity does not affect the ability of aggregates to form since aggregates of equal sizes were obtained using H-2 matched and mismatched combinations. Instead, it may affect the positional arrangement of cells within the aggregates after 24 - 36 hours. Aggregation and positioning mechanisms could therefore be totally different phenomena. Aggregation may be H-2 independent and perhaps occur via non-specific interaction mechanisms which could be triggered or enhanced by H-2 disparity.This was indicated here by increase in PEC spread area and count after interaction with allogeneic H-2 I-A fragments. Positioning may be H-2 influenced, as indicated by the sorting out experiments discussed above. The exact nature of H-2 involvement in positioning mechanisms remains speculative and the tissue - specific interaction mechanisms which result in preferred adhesion between like cells are often discussed, but are similarly elusive despite extensive searches for them. Perhaps H-2 is involved in all of these phenomena, but different subsets of H-2 products are responsible for each type of interaction. If heterogeneity of expression of a particular allele is seen in other cell types as well as pulmonary endothelia as discussed previously (section 8.10), it is also possible that different H-2 products are

expressed to varying extents in different tissues and one could speculate that this could provide a basis for tissue - specific differences, although more evidence would be required to support such an argument.

8.14. Points For Future Investigations.

One of the central questions in immunology concerns the nature of the receptor for H-2 on the cell surface. Speculations as to whether the binding sites for M.H.C. products are also M.H.C. antigens (Greaves, 1975: Snell, 1979) have not been substantiated or revoked by experimental evidence. The nature of the T cell receptor similarly remains elusive, as discussed previously (see section 1.4) although its expression of immunglobulin 'V' region - like genes is becoming more widely accepted (Binz and Wigzell, 1975, 1977: Rajewsky and Eichmann, 1977: Weinberger et al, 1979: Reinherz, 1983).

Ia products have been detected on the surface of T cells by a number of workers (Munro and Taussig, 1975; Tada et al, 1975, 1978; Mozes, 1976; Isac et al, 1977; Katz, 1977a; Marrack and Kappler, 1979; Binz and Wigzell, 1981; Hayes and Hullett, 1982). Whether Ia products are acquired by T cells and subsequently expressed in an antigenically recognisable form as demonstrated by Delovitch (1982), or whether T cells actually synthesize Ia products, is as yet unresolved. Furthermore, there is still some dispute regarding the direct involvement of Ia products in the T cell receptor (Katz, 1977b: Dorf, 1981). In fact, McDevitt and associates have previously reported that anti Ia sera could not block (TG)-A-L antigen binding by T (or B) lymphocytes (McDevitt

et al, 1969, 1972). Munro and Taussig (1975) reported that nonresponsiveness to the synthetic polymers (TG)-A-L or (TG)-Pro-L could be explained either by the inability of nonresponder T cells to produce an antigen - specific helper factor or by the inability of nonresponder B cells to accept such a factor. These investigators postulated two gene control of the immune response to these branched polymers, one gene expressed in helper T cells and the other gene in B cells. Advances in the study of H-2 involvement in immunological interactions await the further elucidation of the molecular mechanisms which operate during T - macrophage, T - B, and T - T cell interactions.

Further work remains to be done to determine whether PEC's absorb other H-2 fragments on their surfaces in addition to Iα. It would be interesting to measure the number of Iα molecules absorbed to determine if saturation can be achieved. The rate of turnover of absorbed Iα molecules at the cell surface could be studied, and the fate of these molecules. For instance, how long do they take to detach from the cell surface, or do they remain bound to the surface until they are internalised ? Can their binding be blocked using monoclonal antibodies or other reagents ? Can different types of allogeneic Iα fragments compete for cell surface binding ? Further investigation is also required into the events taking place after Iα molecules have bound to allogeneic cell surfaces. For instance, is the resulting change a passive or an active event ? Can it be blocked by any enzyme inhibitors ? Experiments attempting to mimic the effects of allogeneic Iα molecules on cell - substrate interactions could also be done.

The possibility of sorting out of endothelial - like cells according to H-2 type is an exciting result which needs a great deal of further work. Whether this is a property solely exhibited by endothelial - like cells, or whether other lines exhibit the same phenomenon, requires clarifying. However, it may be significant that differences within H-2 can somehow give positional information in that cells of one type segregate externally to those of another type. Previous work on the segregation of cells in aggregates has resulted in the formulation of a number of theories on positioning, each of which has been supported by experimental evidence. These will now be considered. (The mechanisms by which cell adhesions take place have been reviewed in length elsewhere and will not be discussed here (Curtis, 1973, 1983).)

Very little work has been done on cell positioning over the last ten years and the hypotheses have remained largely untested since the pioneering work of their proposers. (For historical background, see Curtis, 1978b). Several types of sorting out patterns are seen and it is relevant to consider these here. Firstly, one cell type may entirely enclose another in a typical "sphere - within - a - sphere" arrangement (Steinberg, 1964). Alternatively, several discrete clusters of one tissue type may be found in a mass of the other. Partial enclosure of one tissue by another, or even distinct separate aggregates of each cell or species type have also been recorded. These patterns were reviewed by Curtis (1967). The reproducibility of these patterns for any given mixture of cell types under defined conditions has been assumed, but only partially reported on by Steinberg alone

(1964). Very little of the work has been repeated under the same conditions but rather model systems and techniques have diversified. Weston and Abercrombie (1967) have however reported variation in positioning, but these were not aggregates.

The theoretical explanations of the patterns seen fall into at least five different hypotheses, and these will be discussed in turn.

8.15. Specific Adhesion Theory.

Proposed by Wilson (1907), Galtsoff (1925) and Moscona (1962), this theory states that only cells of the same type will stick together. This is brought about by the binding of complementary molecules on the cell surface. In 1958, Steinberg modified this theory, saying that adhesions between like cells were preferred over adhesions between unlike cells, as he realised that in its initial form, the theory implied that cells in mixed reaggregation cultures would form separate aggregates according to cell or species type. Although this theory explains the adhesion of cells to a pre-existing framework, it does not explain how the framework itself is generated. Steinberg (1958, 1962) and Curtis (1961, 1967) pointed out that during reaggregation, patterns emerge from what appear to be random beginnings and this cannot be explained by specific adhesion. If we consider the sorting out of two cell types, it is quickly apparent that no pattern would emerge due to a greater or lesser degree of specificity.

8.16. Differential Adhesion Hypothesis.

This theory was first proposed by Steinberg in 1964, and has recently been reviewed (Steinberg 1978). It states that cells

sort out according to differences in the strength of intercellular adhesions. The result is that cells rearrange to minimise their total adhesive free energy, ie; so that the system reaches optimal thermodynamic conditions of lowest free surface energy. This was based on the observation that sorting out patterns were homologous with the patterns of contact of two immiscible oil droplets in a third medium. Since interfacial tensions drive these contacts to their resultant patterns we would expect cells to behave in this way. In a mixture of any two cell types, the more cohesive population sorts out internally to the less cohesive. In the case of liver and limb bud, the latter are seen as the most cohesive. The two tissues adopt a sphere within a sphere arrangement with the liver cells taking up the outer position surrounding the limb bud cells. The sphere within a sphere arrangement is an equilibrium configuration in that it can be approached from a different starting configuration. Fusion of a liver aggregate with a limb bud aggregate is followed by envelopment of the limb bud by the liver. From this experimental design, Steinberg arranged large groups of cell types in sequential order. The hierarchy is as follows;

Back epidermis

Limb bud precartilage

Pigmented epithelium of the eye

Heart Ventricle

Neural tube

Liver

In this series each tissue has been found to envelop those preceeding it and can be enveloped by all those following

it. The sphere within a sphere arrangement requires that the heterotypic adhesions (ie; those between different cell types) should be intermediate in strength between the two types of homotypic adhesions, or at least weaker that the weaker homotypic adhesion. Alternatively, if the two cell types are cohesive and motile and heterotypic adhesions are stronger than the average strengths of the homotypic adhesions, the two cell types will mix. Patternings postulated on these theories have all been experimentally realised by Steinberg. These observations imply that there is probably some single graded property shared by all the tissues in the list, which determines the sorting out positions adopted due to the quantitative effects. This theory also explains the adhesion of unlike cells at the beginning of aggregation. Furthermore, it argues against the operation of specific adhesions in sorting out.

Some observations cannot be easily explained by the Differential Adhesion Hypothesis. For instance, Townes and Holtfreter (1955) found that although amphibian mesoderm "sorts out" internally in combination with ectoderm or endoderm, in a tertiary tissue mixture mesoderm lies between endoderm and ectoderm. Harris (1976) has raised a number of questions on the validity of the differential adhesion hypothesis, but these have been countered by Steinberg (see Steinberg 1978 for discussion).

8.17. Timing Hypothesis.

Proposed by Curtis (1961, 1962), this theory states that cell adhesiveness can vary differentially according to cell type. He pointed out that during aggregation, the outer cell type may

become adhesive before the inner cell type and thus 'herd' the cells which are less adhesive at that time into the interior of the aggregate. Consequently, the cells on the interior may not necessarily be of the same type (Curtis, 1967), contrary to the arrangement predicted by the differential adhesion hypothesis (Steinberg 1964) discussed above. In addition, the externally segregating cell type will be the most adhesive initially, whereas according to the differential adhesion hypothesis it would be the least adhesive. The experimental evidence in support of Curtis's hypothesis was obtained using mixtures of amphibian mid - gastrulae cells, or mixtures of various sponge species, in which one cell type had been artificially aged in culture.(See Curtis, 1967, for comments.)

8.18. Chemotaxis.

This theory was originally proposed by Townes and Holtfreter (1955) and Stefanelli and Zachei (1958). They suggested that cell sorting out was a result of concentration gradients of cell products in the aggregate. Although this was theoretically attractive in explaining groupings of cells, none of these workers produced any evidence that chemotaxis did or did not occur in their aggregates. In 1969, Trinkaus and Monahan observed living aggregates of pigmented and non-pigmented cells. They concluded that cells showed no directional movement towards others of the same type and often the converse was seen. In natural aggregation of slime moulds, Bonner (1947) and Shaffer (1957) had previously reported positive chemotaxis, but this was not apparent in their segregation. Furthermore, the existence of a hierarchy of sorting

out cannot be explained by chemotaxis. Lackie and Smith (1980) have shown that chemotaxis involves changes in adhesion and thus the levels of adhesion could be so controlled. A full theoretical discussion that chemotaxis might act in positioning of cells is given in Curtis (1978).

8.19. Interaction - Modulation Hypothesis.

Originally proposed by Curtis and Van de Vyver (1971), this theory states that cells secrete diffusible factors which can interact specifically with a target cell, resulting in its reduced adhesivity. Since they can reduce the target cells ability to stick to any surface to which they would normally adhere, the result of the interaction is non-specific. Thus, the factor controls the level of adhesion of another cell type. Consequently, these factors have been termed Interaction - Modulation Factors (I.M.F.'s). Curtis and de Sousa (1975) investigated the effects of I.M.F.'s on the sorting out of cells in mixed aggregates. They found that when gradients of embryonic chick tissue I.M.F.'s were applied to mixtures of cells, sorting out patterns could be reversed. In mixtures of embryonic chick liver or neural retina, the patterns became randomised. Thus, I.M.F.'s could be viewed as one of the potential candidates in the search for the molecules providing positional information in pattern formation.

Moving away from embryological interactions, further work showed that T lymphocytes produce I.M.F.'s when grown in serum free medium for 1 - 2 hours. This T-I.M.F. reduces the adhesiveness of B lymphocytes and is a soluble fragment of the H-2D product (Curtis and de Sousa, 1975a). If B cells were

pretreated with I.M.F. then injected into syngeneic recipients, or indeed if T-I.M.F. alone was injected even in low doses, B cells would be released from the spleen into the blood. The presence and action of I.M.F.'s in vivo may therefore be an important controlling mechanism in the movement of cells around the body (Curtis, 1978, 1978b, 1979c, Curtis and de Sousa, 1975, Curtis et al, 1979). Most interestingly, Curtis and Davies (1981) have demonstrated that the T cell factor which decreases the adhesiveness of B lymphocytes, also decreases the adhesiveness of other T cells when they are allogeneic at H-2D. Furthermore, the T-I.M.F. was found to be identical in nature to the papain - cleaved fraction of the H-2D product. B lymphocytes were also found to produce I.M.F.'s and these showed restrictions for H-2K or -I-A products in that they could only interact with T cells matched at these loci. These IMF's were injected into mice syngeneic with the source of I.M.F., B cells were found to move from the spleen and into the circulation. If lymphocytes were pretreated with I.M.F. and injected into syngeneic recipients, the localisation of cells in the liver, lymph nodes and small intestine was found to be lower than that found with untreated cells (Davies and Curtis, 1981, Curtis and Davies, 1980). This was the first experimental evidence to strongly implicate histocompatibility antigens in the control of cell adhesion and positioning systems. I.M.F.'s could possibly determine cell position by concentration gradients with cells moving away from the source of the factors. This hypothesis may be supported by the work in this text, as H-2 has been implicated in cell positioning in sorting out experiments, and the binding of H-2 fragments to

the surfaces of allogeneic cells has been shown to non - specifically affect cell adhesivity to substrates. The Altered Self and the Dual Receptor models of CTL interaction mechanisms fail to explain how the binding of allogeneic fragments to cells may be brought about. Indeed, the finding that allogeneic molecules bind to the surface of cells may cast some doubt on the relevance of these explanations to any other than chemically- or virally-modified syngeneic interactions.

Another hypothesis which has recently been suggested is supported by these findings. This hypothesis envisages that H-2 fragments both interact with the cell surface, but a change in cell properties only occurs after an interaction with an allogeneic fragment. This is also partly supported by my own observations on CTL systems. I found that allogeneic fragments could inhibit an altered self response, whereas syngeneic fragments certainly showed no inhibition and in some cases showed enhancement (unpublished observations). These results are however, only preliminary and further work needs to be done to establish that the trends seen were not due to technical variation, although this explanation seems unlikely. It seems that the answers to the questions posed by cellular interactions will probably be based upon observations of immunological phenomena and so it is appropriate that this text should conclude with some final comments on immunological interactions.

8.20. Concluding Remarks.

The ability of an individual to mount a humoral response against a particular antigen is under the control of the M.H.C.

(Katz, 1978). Studies utilising congenic strains of mice established the linkage of responder and nonresponder status to at least two H-2I region genes (Benacerraf and Katz, 1975). The intercellular communication leading to the development of the immune response is similarly M.H.C. controlled. T cells, B cells and macrophages are able to intercommunicate with each other by various means. Whether this intercommunication involves cell contact activity of secreted (or released) molecules, or a combination of cell contact and mediator release, has not yet been definetly established. However, it is known that this intercommunication between T and B cells (T - B cooperation) is generally controlled by I region genes and if these cells differentiate in H-2I - incompatible hosts, cooperation may be prevented (see Katz, 1978).

T helper cells which bear the Ly - 1 phenotype are committed to the recognition of thymus - dependent antigens, in conjunction with I region gene products on macrophages. Similarly, CTL's show specificity for antigen presented in the context of autologous K, D, or L region products. It is still unclear whether antigen and self H-2 products are expressed as one entity in CTL systems (see section 1.4.2.). In the context of antigenic presentation, some interesting work has been done investigating this question. Using F_1 guinea pigs, evidence has been obtained (Benacerraf et al, 1971: Bluestein et al, 1971: Shevach et al, 1973) for an association at the macrophage level, of Ia and antigen, and / or selective expression, at the level of the T lymphocyte, of the receptor for a specific antigen with specific anti Ia receptor. A resolution of this awaits biochemical analysis

of the T cell receptor (for further discussion, see Benacerraf, 1981). Perhaps this will provide an insight into the interactions taking place at the molecular level in these systems.

To date, very little work has been done on T helper restricted activity in CTL responses. However, the demonstration that T cell blasts express increasing amounts of Ia antigens on their surface membranes (Hayes and Bach, 1978) suggested that helper T cell activity for T cell responses could be M.H.C. restricted. Experiments are in progress to resolve this point.

Martz (1980) has argued that during the 'lethal hit" phase of CTL killing, cells are not held together by antigen - receptor binding, but instead this immune recognition triggers another more general non - immunological cell adhesion process which has features in common with other adhesions between types of tissue cells. Since the M.H.C. is involved in the restriction of CTL for targets, this argument relates the M.H.C. with non - immunological cellular adhesiveness and therefore supports older ideas on M.H.C. control of cellular interactions (Bodmer, 1972).

A well - documented example of the involvement of the M.H.C. complex in cellular interactions has been provided by Katz (1978). He has suggested that B - T communication takes place via H-2I encoded surface bound molecules, termed 'cell interaction' (C.I.) molecules and the I region genes controlling them the C.I. genes. He further suggested that when C.I. molecules on T and B cells were genetically identical, binding would take place between them resulting in effective interactions in the development of the humoral response. In conclusion, this mechanism envisages that B - T cooperation has evolved a mechanism for self - recogntion via H-

2I region encoded molecules. Whether C.I. genes and those controlling responsiveness to a specific antigen (Ir genes) are one and the same is still open to speculation. However, it is now thought that cell interactions occur via complementarity rather than by homology as was suggested by Katz's model.

To summarise, the benefits to be gained from studies on the immune response are readily apparent. It may be anticipated that the biological models arising from this work will be useful in explaining other similarly complex systems involving cell interactions, particularly in the subjects of developmental biology and neurobiology. Although more work remains to be done on the mechanisms operating at the molecular level, it is becoming clear that the M.H.C. does in fact play a major role in the control of many types of cellular interactions, thereby supporting the suggestions put forward nearly 20 years ago by Bodmer (1972) and Edelman (1976).

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