STUDIES ON LYMPHOKINE ACTIVITY IN THE AMPHIBIAN *RANA TEMPORARIA*

BY A.J.H. GEARING, BSc.

A Thesis submitted for the degree of PhD.

University of Aston in Birmingham

Submitted in January, 1983

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SUMMARY:

This thesis describes experiments which demonstrate that an anuran amphibian, *Rana temporaria*, is capable of elab orating a range of soluble mediators, analogous to mammalian lymphokines. In vitro stimulation of *Rana temporaria* leucocytes, with both antigens and mitogens, was employed. The supernatants from such stimulated cultures were applied to assay systems, in which their effects on other leucocytes could be determined.

Depending on the circumstances and kinetics of their production, a variety of activities, ascribed in mammals to lymphokines could be demonstrated. These are listed below in terms of their effects on a particular population of cells, and are accompanied by the lymphokine, which is thought to accomplish an equivalent effect in mammals.

1. Activity decreasing random movement of peritoneal cells, Migration inhibition factor or MIF

2. Activity increasing random movement of peritoneal cells, Migration stimulatory factor of MStF

3. Activity increasing glucose uptake by peritoneal cells, Macrophage activating factor or MAF

4. Activity causing directional movement of peritoneal cells, chemotactic factor or CF

5. Activity increasing tritiated thymidine uptake by splenocytes Mitogenic factor or MF

6. Activity decreasing tritiated thymidine uptake by splenocytes Inhibitor of DNA synthesis or IDS

This is the first survey of lymphokine production by an ectothermic vertebrate, and shows that the properties, kinetics and methods of induction of amphibian lymphokines are remarkably similar to their mammalian counterparts. *Rana* MIF was also effective in inhibiting the migration of rat peritoneal cells. This conservation of function suggests that lymphokine production is a fundamental property of vertebrate leucocytes, provides further support for their having a central role to play in *in vivo* immunity; and reveals that in phylogeny, they are present at the amphibian level of evolution.

Key Words: Phylogeny, Immunity, Lymphokines, Amphibia

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CHAPTER ONE

GENERAL INTRODUCTIO N

Recent years have witnessed an upsurge of interest in comparative aspects of immunity. This has been reflected in a steadily increasing number of conferences and symposia on the subject, and the formation of a new International Society of Developmental and Comparative Immunology in 1980. Since 1977, research into the phylogeny of immunity has been given a regular outlet in the Journal of Developmental and Comparative Immunology. Studies on all but the most obscure animal phyla have now been carried out. (Cooper, 1976) (Manning and Turner, 1976) (Solomon, 1981). The results obtained from such investigations have enabled Hildemann to construct five basic levels of immuno-evolution (Hildemann, 1974), which are illustrated in Fig. 1.1.

Occupying the first level, the Protozoa, Porifera, and plants display only a rudimentary type of immunity, in that they are only equipped to recognise "self", and their cells will only associate with like cells, this association occurring by way of complementary cell surface receptors. The phagocytic uptake and destruction of foreign material, seen at this level, is relatively non-specific, and is related to the normal feeding behaviour of the cells, where, for example, the differentiation between food items and inert particles is possible.

At the second level, which includes the tunicates and coelentrates, there exist cells which specialize in the recognition and destruction of "non-self" materials. . The rejection of both xenografts and allografts is a consistent feature of animals in this group. (Van der Vyver, 1980).

Further differentiation of leucocytes occurs in level three which includes the protostome annelids, molluscs, and arthropods, and also the deuterostome echinoderms. Here, efficient allograft responses with specific memory have been induced. Animals at this level also employ a range of strategies to inactivate or destroy pathogenic organisms, including the formation of granulomatous lesions, and the use of various opsonizing factors to facilitate phagocytosis (Solomon and Horton, 1977).

The vertebrate level of evolution is characterized by the appearance of immunoglobulin, as both a cell surface receptor, and as an effector molecule. The functional differentiation into 'T' and 'B'-like lymphocytes has been described in the fourth level, which includes the fish, reptiles and primitive amphibia. These groups display integrated cell-mediated and humoral immune responses, but their antibody production is limited to a single species of immunoglobulin, similar to mammalian IgM, which may exist in high or low molecular weight forms (Cooper, 1976) although reptiles also possess a low MW Ig N.

The immune system becomes most complex in the fifth level, which encompasses the advanced amphibia, birds and mammals. Here the further differentiation of 'T' and 'B' cells into a number of interacting subsets has been more fully demonstrated, and the production of up to five major classes of antibody is observed (Hood, Weissman and Wood, 1978). Despite the complexity of the immune system at this level of evolution, it has, paradoxically, become the best characterized and understood. This is to a large extent due to the routine use of mice, rats, and guinea pigs in immunological studies, and the fundamental importance of clinical immunology, which guides both effort and money towards mammalian or human orientated research.

The prevailing view of the mammalian immune system holds that the outcome of any antigenic disturbance to that system is dependent on the results of interaction between the antigen and the 'T' helper (T_h) and the 'T' suppressor (T_s) lymphocyte circuits. Depending on the concentration, route of introduction, and physical state of the antigen, then either 'T' cells of the helper lineage or those of the suppressor lineage may be activated. The effect of T_h cell activation is to give an immune response which leads to the destruction or inactivation of the antigenic stimulus. If, however, antigen presentation is such that T_s cells are stimulated, then they act to selectively prevent antigen specific T_h cells from functioning and hence to prevent the subsequent immune response, rendering the animal tolerant to that antigen. The balance between T_h and T_s cell function is thought to represent a homeostatic feedback circuit which controls the immune response of an animal to any particular antigen.

Following the demonstration that both T_h and T_s functions depend on more than one type of T_h and T_s cell (Gershon and Cantor, 1979), and that cell-cell interactions play a vital part in any immune response (Niederhuber, 1979), a major theme in modern immunology has been to elucidate the mechanisms by which cells interact.

It has become apparent that lymphoid cells can communicate with each other by way of a series of soluble mediators. Historically the study of such mediators began with initial observations in 1932 that cells from spleen fragments of tuberculous guinea pigs could be inhibited in their migration if these cells were first exposed <u>in vitro</u> to old tuberculin antigen

(Rich and Lewis, 1932). Subsequently, little interest was shown in this phenomenon until the 1960s when a soluble factor was discovered which produced by sensitized lymphocytes following in vitro antigen challenge, inhibited the migration of peritoneal exudate cells (PEC) invitro (Bloom and Bennett, 1966) (David, 1966). This factor was named migration inhibition factor or MIF. This initial report was followed in the late 1960s by the demonstration of several other factors affecting both macrophage function, e.g. macrophage activating factor, or MAF, which stimulated a variety of macrophage responses, e.g. lymphocyte mitogenic factor, or LMF, which increased the proliferation of lymphocytes (Maini, Bryceson, Wolstencroft and Dumonde, 1969). Both MAF and LMF were produced following antigenic challenge of sensitized lymphocytes in vitro. A different "blastogenic factor", or BF, which stimulated the division of allogenic lymphocytes, was demonstrated in culture of alloantigen activated lymphocytes (Gordon and Maclean, 1965), (Kasakura and Lowenstein, 1965). At about this time a lymphocyte derived factor which was cytotoxic for other cells was also described (Ruddle and Waksman, 1967). In 1969, Dumonde and Co-workers proposed the generic name "lymphokine" for such mediators, and defined them as non-antibody products of lymphocyte activation, which affect the behaviour of other cell types (Dumonde, Wolstencroft, Panay, Matthew, Morley and Howson, 1969).

The 1970s saw a vast increase in the number of reports of mediators produced by lymphocytes, (Pick and Turk , 1971), (Rocklin, 1976)(a)and now well over one hundred lymphokine activities have been described (Waksman and Namba, 1976). Although it was originally assumed that 'T' lymphocytes were the source of lymphokines, it is now known that 'B' cells,

macrophages and even certain non-lymphoid cells can produce similar, or identical mediators (Waksman, 1979). The name lymphokine remains restricted to lymphocyte derived mediators, macrophage derived factors are called monokines, and mediators from other cell types are called cytokines (Bigazzi, 1979).

Lymphokines are only produced in picogram quantities in normal cell cultures, and hence they are routinely defined by their activity in bioassays (Morley, Wolstencroft and Dumonde, 1978). The nature of the appropriate bioassay is generally reflected in the name given to a particular lymphokine. Thus, MIFs act to inhibit the migration of a particular cell type, and MAF to activate a variety of macrophage functions. In general, lymphokines are glycoprotein molecules of between 10-100 $\times 10^3$ Daltons, and are produced by lymphocytes which have been stimulated, (normally by antigen or mitogen), to make the transition between the G_0 and G_1 states of the cell cycle (Waksman, 1979). Lymphokines have been categorized into two groups consisting of 1) factors affecting inflammatory responses and, 2) those affecting the proliferation or differentiation of other lymphoid cells (Waksman, 1979) (see Fig. 1.2. By far the greater number of factors fall into the second category of regulatory lymphokines, these include many helper and suppressor factors, which can be antigen specific or non-specific, and restricted or non-restricted in their interactions with cells on the basis of their major histocompatibility complex (MHC) type (Altman, 1980), (Feldman and Kontiainen, 1981). The inflammatory lymphokines, including MIF, MAF and chemotactic factors (CF), although fewer in number, have received more attention historically and have been better characterized

(Adelman, Hammond, Cohen and Dvorak, 1979). Inflammatory lymphokine production has long been used as an *in vitro* correlate of *in vivo* cell mediated immunity, as in delayed type hypersensitivity reactions (George and Vaughan, 1962), or in allograft rejection (Friedman, 1971) An *in vivo* role for lymphokines in such reactions has been suggested. Thus injections of a crude lymphokine containing supernatant can cause the development of typical DTH lesions in guinea pigs, and injection of anti-lymphokine serum can abrogate antigen-induced DTH skin reactions (Neta and Salvin, 1981). Although the situation is still unclear, it is generally assumed that lymphokines play a central role in *in vivo* cell interactions, see Fig. 1.3.

The Amphibia have long been recognised as occupying a pivotal position in the evolutionary sequence of vertebrate immunity (Cohen, 1975). The more primitive Apoda and Urodela, display a rather sluggish immune response, typical of Hildemann's fourth level of immunoevolution, with a single class of immunoglobulin, chronic allograft rejection capability, and poorly developed lymphoid organ architecture, whilst the Anura display an altogether more sophisticated level five, type of immune system (reviewed in Manning and Turner, 1976) and (Cooper, 1976). Anurans can produce two types of antibody molecule, IgM and IgRAA, the kinetics of their release paralleling the mammalian response, with a secondary antigen challenge resulting in a switch from the IgM to the IgRAA antibody (Geczy, Green and Steiner, 1973) (Atwell and Marchalonis, 1976). The Anura also display acute allograft responses, indicating the presence of a mammalian type of MHC (Cohen, 1971). Both thymus dependent and independent subsets of lymphocytes can be demonstrated by the use of 'T' and 'B' cell mitogens (Manning, Donnelly and Cohen,

1976) nylon wool separation (Blomberg, Bernard and Du Pasquier, 1980) monoclonal antibody (Bleicher and Cohen, 1981) and in particular, the use of neo-natal thymectomy. The ease with with tadpoles of Xenopus laevis can be thymectomized very soon after fertilization, has enabled many authors to demonstrate that following such operations, 'T' cell dependent responses such as, allograft rejection (Horton and Manning, 1972) (Rimmer and Horton, 1977), antibody production to thymus dependent antigens (Horton and Manning, 1974) (Horton, Rimmer and Horton, 1976) (Horton, Rimmer and Horton, 1977) and both 'T' cell mitogen and mixed lymphocyte responses (Du Pasquier and Horton, 1976) are abrogated. The thymectomized Xenopus has become a classic 'T' cell depleted model, equivalent to the nude mouse in its usefulness to amphibian immunology. The recent development of a series of clones of histocompatible Xenopus (Kobel and Du Pasquier, 1975) and improvements in tissue culture techniques for amphibian cells, has enabled the genetic control of cell interactions, both in vitro and in vivo, to be examined. Du Pasquier and co-workers have shown that Xenopus 'T' and 'B' cells collaborate in vitro to give an antibody response (Blomberg, Bernard and Du Pasquier, 1980), and that for secondary Ig 'G' production, that the 'T' and 'B' cells must be from animals sharing at least one allele of the Xenopus MHC (Bernard, Bordmann, Blomberg and Du Pasquier, 1981). Such in vitro restriction of interaction has not yet been confirmed in vivo. The implantation of MHC disparate thymus into thymectomized toads was shown to restore both IgM and Ig'G' antibody responsiveness (Du Pasquier and Horton, 1982). The mechanism of this restoration is currently under investigation (Horton, personal communication).

The effects of thymectomy on classically 'T' cell dependent responses, and the existence of 'T' cells which co-operate with 'B' cells in the production of antibody, is evidence that the T_h subset

is present in anurans. Such direct evidence for the T_s subset is not as extensive. Specific tolerance can be induced to a variety of antigens (Marchalonis and Germain, 1980), and the period of metamorphosis has been shown to be a time when tolerance is easily achieved to alloantigens (Du Pasquier and Bernard, 1980). Such tolerance could be transferred to normal animals by lymphocytes from tolerant individuals. In vitro experiments have implicated the thymus as a source of suppressive activity, co-culture of antigen sensitized spleen and thymus fragments causing reduced splenic antigen-binding cell and antibody levels compared to levels in cultures of spleen fragments alone (Ruben, Mette, Cochran and Edwards, 1980). A thymus derived suppressor is also indicated for the axolotl, in which thymectomy causes an increase in antibody levels towards injected horse erythrocytes (Charlemagne, 1979), Charlemagne suggests that this is due to the removal of suppressor 'T' cells, which normally keep the response in check.

Whilst studies on the phylogeny of immunity and investigative work on lymphokines have proceeded apace for perhaps two decades, for the most part the two areas have progressed independently of each other. This thesis endeavours to integrate two fields of immunology which have gradually assumed an increasing interest and importance in their own right. Although at a gross functional level, it would appear that the immune capabilities of anurans are similar in many respects to those of mammals, there is little information, at present, concerning the way in which the cells of the amphibian immune system interact with one another. Despite the importance of lymphokines in mammalian immune reactions, information on their significance in other taxa is very restricted.

The work presented in this thesis represents an initial attempt to investigate the existence of lymphokine activities at the level of the amphibian, and compares such findings with those reported for mammals. The advanced anuran, Rana temporaria, was chosen for these studies, due to its availability and ease of husbandry.

This thesis is sub-divided into chapters as follows. Chapter two details the rationale employed in the investigations of lymphokine production and function which are described in the subsequent chapters. Chapter three is concerned with lymphokines affecting the random migration of peritoneal exudate cells; chapter four investigates lymphokines affecting directional migration of PEC and chapter five details experiments which examine lymphokines which affect the proliferation of spleen cells. The final chapter integrates the findings in this thesis with other reports of lymphokine activity in non-mammalian vertebrates, and discusses their significance with regard to the study of both amphibian immunity, and the evolution of the immune system.

Level 5	Complex immunoregulation Ig specialization	T/B lymphocyte subsets	Birds, Mammals (Reptiles)
			Amphibia
Level 4	Integrated cell mediated and humoral immunity	B lymphocytes	Fish
Level 3	Cell mediated immunity with memory	Granulocytes and "T" lymphocytes	Tunicates, Echinoderms; molluscs, annelids, arthropods
Level 2	Non-self recognition	Differentiated leucocytes	coelenterates
Level 1	Self recognition	Epithelial/ cell surface receptors	sponges, plants, protozoa

Fig. 1.1 Levels of immunoevolution (modified from Hildemann, 1974).

Fig. 1.2 Summary of mammalian lymphokine activities

Inflammatory	Regulatory
Migration inhibition factor MIF	Mitogenic factor MF
Migration stimulatory factor MStF	Helper factors *
Macrophage activating factor MAF	Suppressor factors *
Chemotactic factors CF	Interleukin 2
Cytotoxic factors	Colony stimulating factors
Interferons	Osteoclast activating factors
Vascular permeability factors	

* Both helper and suppresor factors may be antigen specific, or non-specificin nature, and MHC restricted or non-restricted in their interaction withtheir target cells.

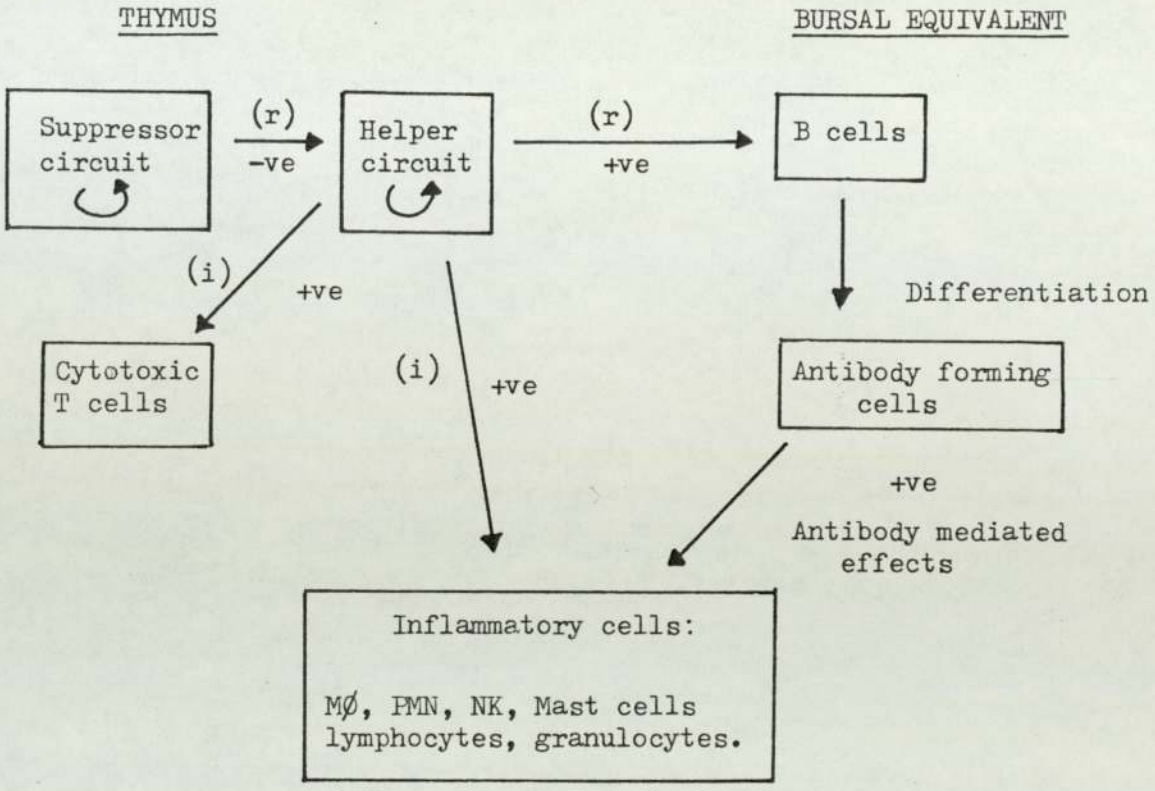


Fig. 1.3 Simplified diagram of lymphokine involvement in mammalian immunity. Regulatory lymphokines are indicated by (r), and inflammatory lymphokines by (i). Abbreviations are as given in the appendix.

CHAPTER TWO

ASSESSMENT OF LYMPHOKINE ACTIVITY

By definition lymphokines are soluble, non-antibody products of lymphocyte activation which produce a range of biological effects on the appropriate target cells. Classically, they have been studied and defined on the basis of their biological action *in vitro*, and a wide range of test systems for the study of lymphokines has now been developed.

In beginning to study lymphokine activity in the amphibian, the present work rests heavily upon the development and modification of bio-assay techniques which have been used extensively in mammalian cell systems. The enormous range of biological activities which have been ascribed to different lymphokines precluded a study which encompassed the whole range of such activities. It was clear from the outset that a more restricted approach was necessary. The work presented in this thesis is founded on three test systems which are derived from established mammalian experimental protocols. These systems measure:

1) Inhibition of cellular migration *in vitro* (Chapter 3)

2) Directional cellular migration *in vitro* (Chapter 4)

3) Stimulation of DNA synthesis by lymphocytes *in vitro* (Chapter 5)

The choice of these three systems rests upon a number of important considerations. Firstly, the basic experimental protocols for such work are well-established and well-documented in mammalian systems where they can be utilised to produce consistent results. Secondly,

it was felt that the three systems employed would illustrate both "in hibitory" and "stimulatory" biological effects. (Reliance on the measurement of "inhibitory" effects alone cannot preclude the possibility that so called "biological effects" are only a consequence of toxicity). Thirdly, the activities described (in mammals) may be induced by lymphocyte activation with both antigens and mitogens, and would therefore provide a future opportunity for the investigation of lymphokine production by different lymphocyte sub-sets classified on the basis of their activation, by T dependent and T independent mitogens. Finally, the activities selected are not MHC dependent (in mammals) and do not rely upon the use of genetically identical animals.

Whilst details of antigen dose, culture conditions and experimental assay systems will be described fully in the appropriate chapters, it was felt that it would be useful at this stage to describe in general terms the basic experimental rationale employed for the study of lymphokines. This is partly to avoid the necessity for tedious repetition in subsequent chapters and also to describe the general considerations which are involved in the setting up of experiments to assay lymphokine activity.

Throughout this thesis, two basic approaches are employed which for the sake of convenience, will be referred to as 'direct' and 'indirect' assay systems. In the former the stimulus (antigen or mitogen), the lymphokine producing cells, and the cells which respond to the lymphokine are contained in the same culture vessel. Thus in Chapter Three, for example, a population of peritoneal exudate cells containing lymphocytes (lymphokine producers), monocytes and polymorphonuclear cells (responder cells) are allowed to migrate in the presence of the antigenic stimulus for lymphokine production. The migration of the whole cell population is measured in order to determine any lymphokine induced effect. The advantages

of a 'direct' system, such as the ease of setting up the assay system in a single step, must be weighed against the disadvantages which include the limited information which can be drawn from such experiments. For instance, any inhibition of migration cannot be ascribed solely to the production of soluble factors, since effects based on direct 'producer cell' and 'responder cell' contact cannot be precluded.

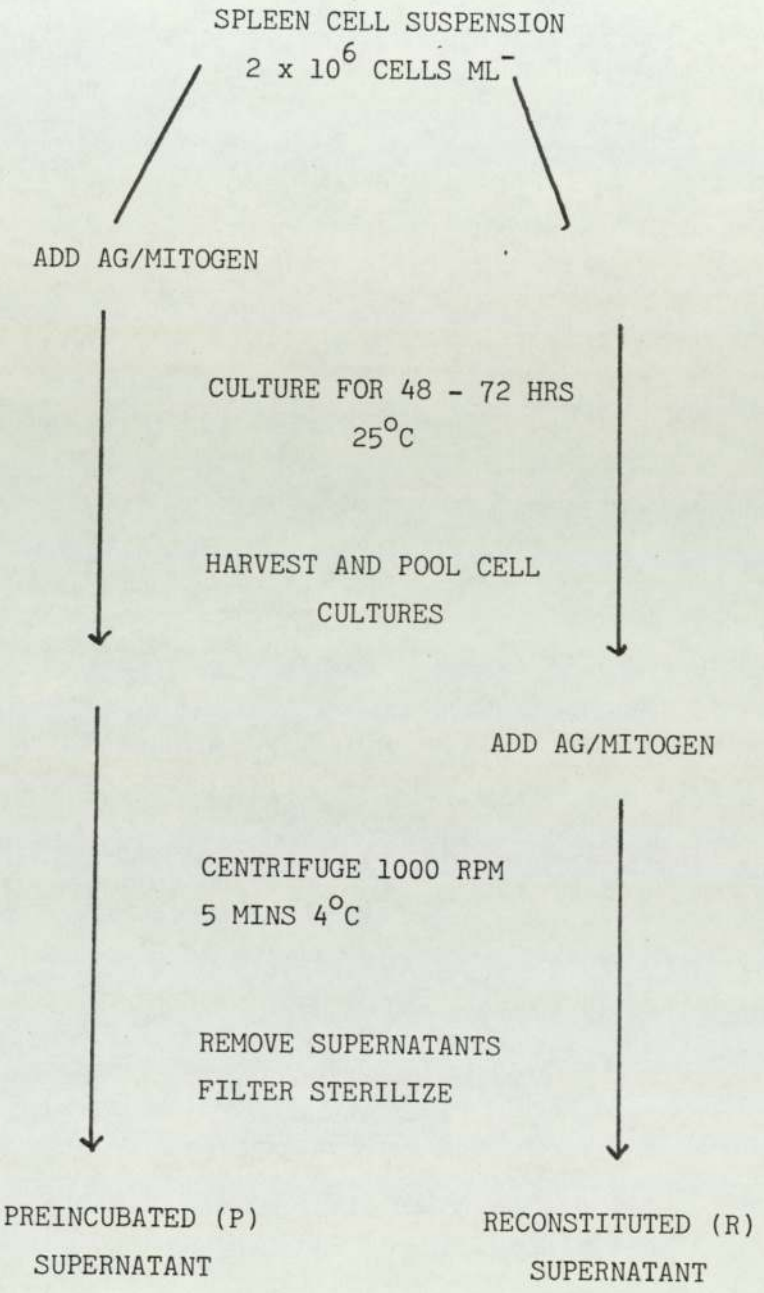
The use of an "indirect" assay system overcomes the limitations outlined above. Here the cells which produce the lymphokine are exposed to the activating stimulus (antigen or mitogen) in culture and a cell free supernatant is prepared which contains any soluble products released as a result of lymphocyte activation. The supernatant (containing putative lymphokine activity) can now be applied to a separate assay system containing the responder cells and its biological effects assayed. The advantages offered by the "indirect" method include the obviation of direct cell-contact effects and also the facility for fractionation and biochemical analysis of the supernatant prior to addition to the responder cells. The general methodology employed for production of test and control supernatants in the "indirect" lymphokine assay is outlined in Figure 2.1 Lymphokine producing cells (normally spleen cells) are stimulated in vitro with antigen following in vitro priming. (Mitogens are added directly to primary spleen cell cultures). Following incubation with antigen or mitogen the culture supernatant is removed and after centrifugation, is filter sterilized and may be stored at - 20°C prior to use. Culture supernatants prepared in this way as a putative lymphokine source are referred to throughout the thesis as "preincubated" (P) supernatants (i.e. culture supernatants derived from preincubation of lymphocytes with antigen or mitogen).

A second control aliquot of spleen cells is cultured under conditions similar to those described above, but antigen or mitogen is only added at the end of the culture period. The culture supernatant is collected as described above, and only then is an equivalent amount of antigen or mitogen added to "reconstitute" the control (R) supernatant. Under this protocol both P and R supernatants contain the same amount of antigen or mitogen; the same general by-products of culture; but only the P supernatant will contain products released as a consequence of antigenic or mitogenic stimulation of lymphocytes. Appropriate comparison of P and R supernatants will therefore demonstrate the requirement for lymphocyte activation (by antigen or mitogen).

It is predicted that (if lymphokines are present) the P supernatant will give rise to a significantly higher level of the appropriate "biological activity" (e.g. migration; inhibition; chemotaxis: increased 3HT uptake) than the R supernatant.

The demonstration of significant differences in the level of responses in the three chosen test systems to P and R supernatants is the aim of the next three chapters.

FIG.2.1 PROCEDURE FOR THE GENERATION OF SPLEEN CELL CULTURE P AND R SUPERNATANTS



CHAPTER THREE

STUDIES ON ANTIGEN AND MITOGEN INDUCED FACTORS AFFECTING THE RANDOM MIGRATION OF LEUCOCYTES

Introduction

Since its original description in 1962 by George and Vaughan (George and Vaughan 1962) macrophage migration inhibition factor (MIF or MMIF) has been joined by other lymphokines which affect the migration of various lymphoid cell types. Thus, factors stimulating the migration of macrophages, (migration enhancement factor or MEF (Weisbart, Bluestone, Goldberg, and Pearson, 1974) and migration stimulation factor MStF (Aaskov and Anthony, 1976)), and also a factor which inhibits the migration of polymorphonuclear leucocytes, (PMN-MIF or LIF (Rocklin, 1978)), have been described.

In view of the vital role that lymphokines are thought to play in the endotherm immune system, it is perhaps surprising that their significance at the amphibian level of evolution has received very little attention to date. Direct evidence for lymphokine activity in the Amphibia has been restricted to studies of leucocyte migration inhibition. Tahan and Jurd have demonstrated migration inhibition of antigen sensitized splenocytes, by the sensitizing antigen, in the urodele Ambystoma mexicanum (Tahan and Jurd, 1979). A similar result was obtained by Drössler and Ambrosius with the anuran Bufo bufo (Drössler and Ambrosius, 1972). They showed that the migration of antigen sensitized peritoneal exudate cells, and splenocytes, was inhibited by the sensitizing antigen. In addition to these demonstrations of migration inhibitory activity, Sipka and co-workers, have shown that in Rana esculenta, the injection of a sensitizing antigen into the peritoneal cavity of sensitized

animals causes a marked reduction in the number of macrophages (Sipka, Boldogh and Szilagyi, 1977). This macrophage disappearance reaction (MDR) is known, in mammals, to be caused by MIF, and indeed Sipka has shown that a rabbit lymphokine preparation, containing MIF activity, will also cause a MDR in Rana esculenta.

The paucity of information on the existence of lymphokines in the Amphibia, combined with the extensive background of information on lymphokines affecting cell migration in endotherms, has prompted this investigation of such factors in the anuran, Rana temporaria. The work presented in this chapter has investigated the problem, by examining the effects of both antigen and mitogen challenge on the migration of PEC in a direct assay. The antigen specificity and in vitro dose response characteristics of the phenomenon have also been characterized. In addition, the production of factors,(by antigen or mitogen stimulated splenocytes) which affect peritoneal cell migration has been investigated in an indirect assay. The kinetics of the reactions have been studied, and have been compared with other aspects of immune reactivity (serum antibody titres and rosette forming cell numbers). The physico chemical nature of the mediators involved and their interactions with their target cells have also been studied, by the use of sugar inhibition and sephadex fractionation techniques. The cross species activity of a Rana MIF preparation was also determined

Materials and Methods

Animals:

Adult Rana temporaria of 45-80 mm snout-vent length, were obtained from the Frog Farm, Co. Meath, Eire. Frogs were maintained in groups of 4 - 6 at approximately 20°C, in tanks arranged to give a constant through flow of shallow water. They

were fed on mealworms or earthworms twice weekly.

Antigen Preparation and Immunization:

Sheep red blood cells (SRBC), in Alsever's solution (flow labs) were washed three times in saline, and resuspended in a 10% v/v suspension in saline for injection. Formalinized SRBC (FSRBC) were prepared by the dropwise addition of 40% formalin (BDH) to a 10% v/v suspension of SRBC in saline to give a final concentration of 3% formalin. FSRBC were stored at $4^{o}C$ and were washed three times with saline before injection as a 10% v/v suspension in saline. Lyophilized human gamma globulin (HGG) (Flow labs) was dissolved in saline to a final concentration of 2 mg ml^{-1}.

Groups of from 4 - 6 animals were immunized by injection of 0.2ml of antigen preparation per 40g body weight, via the intra peritoneal route. Control animals received an equivalent volume of saline only.

Tissue Culture Medium:

Amphibian tissue culture medium was prepared by modification of either Liebowitz or Iscoves medium (Flow labs). The formulation was as follows: to 66ml full strength medium was added 33ml double distilled water, this gave an amphibian tonicity medium, buffered by either Zwitterion action (LIS) or by Hepes (Iscoves). A further 2ml of Penicillin and Streptomycin and 1ml of 2mM L Glutamine was also added to each 100ml of Amphibian medium.

Foetal calf serum (FCS) was routinely added, as 1 ml of heat inactivated FCS per 100 ml medium. Heat inactivation

(56°C - 30 mins). served to remove any complement components present. In some experiments the FCS was omitted from the medium to study whether FCS had any effect on the cell migration (Fox, Gregory and Feldmann, 1974)

Preparation of Spleen Cells for Culture:

Spleens were removed aseptically and teased apart in tissue culture medium on ice. The suspension was transferred to a 15 ml conical test tube, and allowed to stand on ice for 5 minutes to allow tissue debris to settle. The single cell suspension was then removed to a 15 ml graduated centrifuge tube and the cells washed three times in tissue culture medium by centrifugation at 600 rpm for 7 minutes at 4°C. The viability of the cells was determined by trypan blue dye exclusion. The cellular concentration was adjusted to 2×10^6 viable leucocytes ml^{-1}. Cell viability was usually in excess of 95%.

Harvesting Peritoneal Exudate Cells:

Animals were given an intraperitoneal injection of 1% potato starch or thioglycollate medium in saline 3 - 4 days prior to harvesting. This procedure has been shown to increase the cell-yield from the peritoneal cavity (Stuart, Habshaw and Davidson, 1978) Thioglycollate was preferred because it caused less cellular clumping than starch although it was found to give an exudate richer in PMN. To harvest peritoneal cells, animals were anaesthetized in a solution of tricaine Methane Sulphanate (1:4000 dilution) MS 222 Sigma and swabbed with 70% alcohol. A ventral incision was made in the skin from a point immediately anterior to the cloaca to the sternum. The skin was reflected, and 4 - 6 ml of cooled tissue culture medium was injected into the peritoneal cavity. The body wall was gently massaged to bring the peritoneal cells into suspension and the exudate removed using a syringe with a

21 swg needle. All suspensions were transferred to 5 ml heparinised sterile tubes (TEKLAB) and kept on ice. The cells were then washed three times (in tissue culture medium) by centrifugation (at 600 rpm for 7 minutes) at $4^{o}C$ Cells from at least 6 animals were pooled for each experiment. The washed pooled cells were counted in a haemocytometer and their concentrations adjusted to 2×10^{7} viable leucocytes ml^{-1} as assessed by trypan blue dye exclusion.

Immunocytoadherance Assays:

In order to assess the efficacy of the immunizing injections, the number of cells reactive to SRBC or FSRBC, was determined using the method of Ruben and others (Ruben, Van Der Hoven and Dutton, 1973). 50 μl of a 2×10^{6} leucocyte ml^{-1} cell suspension was mixed with 10 μl of a 1% RBC suspension and incubated overnight at $4^{o}C$. The number of leucocytes binding the RBC and forming rosettes was determined by counting in a haemocytometer and was expressed as the number of rosette forming cells (RFC) per 10^{6} leucocytes.

Serology:

To determine the extent of the humoral immune reaction, serum antibody titres were measured, following RBC injections. Blood was collected by cardiac puncture and, after clotting for two hours at room temperature, serum was removed and heated at $56^{o}C$ for 30 minutes to remove complement and stored at $-20^{o}C$.

Haemagglutinating antibody levels were determined using a microhaemagglutination test performed in microtitration trays (Flow) 25 μl of serum (serially diluted) was incubated for 2 hours with 25 μ l of 1% SRBC. The maximum dilution of serum which agglutinated the RBC was taken as the titre of agglutinating antibody, and was expressed as - $\log_2$ titre.

The Capillary Tube Migration Inhibition Assay:

The assay employed was a modification of George and Vaughan's original method (George and Vaughan, 1962). Peritoneal exudate cells (PEC) were taken up into capillary tubes which were sealed at one end with dental plasticine. The sealed capillaries were placed vertically into flat bottomed tubes and centrifuged at 600 rpm for 7 minutes at 4°C, in order to pack the cells onto the plasticine plug. The capillary tubes were then cut at the inter face of the cell pellet and the culture medium above. The resulting sections of tube containing the cell pellets were transferred to individual wells of leucocyte migration trays (Sterilin). The tubes were anchored to the floor of the wells by a spot of sterile vaseline. A coverslip was used to seal each well, and trays were incubated at 25°C on a level flat-bed incubator.

The extent of cell migration was routinely measured at 18 hours, by placing each tray in a photographic plate enlarger and projecting the image of the migrating cells onto graph paper. The outline of the migration fan was carefully traced onto graph paper, and the area determined by counting the squares enclosed. A magnification of 7.5 times was employed.

Initial experiments employed haematocrit tubes of 80μl capacity, but in later work 10μl microcaps (Drummond) were used to increase the number of replicates from a given number of cells. This micromethod described by Federlin and co-workers (Federlin, Maini, Russel, and Dumonde, 1971) was found to give identical migration inhibition values when compared with the 80μl method, and was particularly useful when dealing with small numbers of cells.

The Direct Assay:

In the direct assay, the antigenic or mitogenic stimulus (for producing migration inhibitory factors) was applied directly to the peritoneal cells in the migration wells. Thus antigen sensitized cells were allowed to migrate into culture medium containing antigen, or non-sensitized cells were allowed to migrate into mitogen supplemented medium. The extent of the inhibition of migration, caused by antigen or mitogen, was determined by comparing the mean migration areas in supplemented/with control, non supplemented wells. At least four replicates were used to give a mean migration area. The degree of migration inhibition was then expressed as a % migration inhibition determined using the following formula:

$$\% \text{ migration inhibition} = 100 - \frac{\text{Mean Migration area with antigen or mitogen}}{\text{Mean migration area in control}} \times 100$$

Although a 20% migration inhibition is usually considered significant, the significance of the difference in migration areas, between control and supplemented wells, was determined using students 't' test.

Experimental Design:

a) Antigen induced migration inhibition

The antigen induced migration inhibition response was characterized in a number of ways.

i) Antigen specificity: 21 days following immunization with either HGG or FSRBC, the migration of peritoneal cells was measured in the presence of antigens. HGG sensitized cells were incubated with either HGG or human serum albumin(HSA), and FSRBC sensitized cells with FSRBC or formalinized rat red blood cells(FRRBC).

The effect of the antigens on non-sensitized cells was also determined. Antigens were added as either 10μl of 1% erythrocyte suspension per ml of incubation medium, a concentration deemed optimal by Jayaraman and others, (Jayaraman, Mohan, and Muthukkaruppan, 1979), or as 100μg of protein per ml of medium. This concentration was found in preliminary experiments to give maximal inhibition of migration for HGG sensitized PEC (Rimmer and Gearing, 1980).

ii) Chronological development of the response:
The antigen-specific migration inhibition response following HGG: FSRBC: 10% SRBC or 0.01% SRBC injection was also investigated at different times up to 21 days post-immunization.

iii) Kinetics of the migration inhibition reaction in vitro: For a number of experiments, migration areas of antigen-stimulated and control cells were determined after 1, 18, 42 and 66 hours of incubation

iv) The effect of FCS supplementation: The effect of having either 1% FCS or no FCS in the culture medium was investigated on the antigen-specific migration inhibition response to both HGG and FSRBC.

v) The effect of sugar supplementation: The migration inhibition response to HGG and FSRBC was also studied with regard to the effect of sugars. (Remold 1973). Both α - L - fucose and β- D - galactose were added to the migration wells to yield a final concentration of 0.01 M

b) Mitogen induced migration inhibition

Both T and B cell mitogens were employed to determine their effects on cell migration. The mitogens were added to a final

concentration of either 50 μg ml^{-1} PHA, 200 μg ml^{-1} LPS or 1μg ml^{-1} con A. These concentrations were used as they were found to be optimal for inducing spleen cell proliferation (see Chapter Five). The effects of α - L - fucose, on the mitogen induced migration inhibition reaction, was also investigated, using 0.01 M final concentration.

The Indirect Assay:

This assay was first described by Bloom and Bennett in 1966 (Bloom and Bennett, 1966). As described in Chapter Two, antigen or mitogen stimulation was applied to spleen cells, and the effects of the P or R supernatants on the migration of normal peritoneal cells was determined. The P and R supernatants, generated as indicated below, differ in that only the P supernatant contains the products of stimulated spleen cells. P & R are compared for their effects on cell migration using a % migration inhibition calculated using the following formula:

$$\% \text{ migration inhibition} = 100 - \frac{\text{Mean Migration area in P Supernatant}}{\text{Mean Migration area in R Supernatant}} \times 100$$

The statistical significance of any inhibition was determined using Student's 't' test as for the direct assay.

Generation of Spleen Cell Culture Supernatants:

A microculture method was used to assess the proliferation of amphibian cells (See Chapter Five). 100 μl of washed splenocytes (2 x 10^6 cells ml^{-1}) was added to individual wells of microtitration plates (V shaped well, Flow Labs) and cultured at 25^{o}C in a humid incubator. Spleen cells from different animals were plated out separately, and were each divided into two groups. One group was cultured in the presen ce of antigen or mitogen (Preincubated culture), the other was maintained in culture medium

alone. After 72 hours of incubation (48 hours for con A cultures) the cultures were harvested, the whole cell suspension being transferred to test tubes at 4°C. At this stage all the preincubated cultures were pooled. The cultures maintained in medium alone were also pooled, and now received an equivalent amount of antigen or mitogen, to that employed in the preincubated cultures, these cultures were termed, "reconstituted".

Both cell suspensions were immediately centrifuged at 600g for 5 minutes at 4°C. The preincubated P, and reconstituted R, culture supernatants were removed and filter-sterilized, using 0.22 μm cellulosic filters prior to storage at - 20°C. con A induced supernatants were also subjected to absorption for 5 minutes with a 10% v/v suspension of sephadex G100 before sterilization. This procedure has been shown to remove con A from culture medium by binding to the sephadex beads (Remold 1973).

In the generation of supernatants, antigens were again added as either 10 μl of 1% erythrocyte suspension or as 100 μg of HGG per ml of culture medium in the microtitration plate wells. con A was used at a final concentration of 1 μg ml^{-1}.

Supernatants were added to migration assay wells to 1:4 dilution, unless otherwise specified.

Fractionation of Supernatants:

In an initial attempt at physicochemical characterization of any migration inhibitory activity present, two batches of con A P and R supernatant were separated by gel filtration through sephadex G100 - 120 in a Pharmacia column. The column was run at a flow rate of 0.5 ml per minute at 20°C +/- 1°C, and was calibrated using the molecular weight markers dextran blue (2×10^6 Daltons), bovine serum albumin (67,000 Daltons) and lysozyme (14,000 Daltons).

3 ml of both P and R supernatants were lyophilized using a freeze drier, and the lyophilized samples were then reconstituted to 1ml volume with distilled water. The smaller volume was used in order to achieve better resolution on the column. Fractions I to IV eluting in the molecular weight ranges given below, were collected:

Fraction	MW Range (Daltons)
I	>50,000
II	27 - 50,000
III	16 - 27,000
IV	9 - 16,000

Each fraction containing 9 ml of fluid, was lyophilized again, and reconstituted to 3 ml with distilled water. As the running buffer used in the column was a 1/3 dilution of amphibian phosphate buffered saline, this gave a final solution of amphibian tonicity. Each fraction was filter sterilized, and stored at - 20°C.

Fractions of supernatant were added to migration assay wells at a dilution of 1:2.

Experimental design:

a) Antigen induced Supernatants

Supernatants were obtained from spleen cells challenged with a variety of antigens at different times following antigen injection. The following P + R supernatants were compared for their effects on the migration of non sensitized peritoneal exudate cells:

i) Three different batches of supernatant from animals immunized 21 days previously with SRBC.

ii) Two different batches of supernatant from animals immunized 14 days previously, and one batch of animals immunized 7 days previously, with HGG.

iii) Two different batches of supernatant from animals immunized 14 days previously, and one batch immunized 7 days previously, with FSRBC.

b) Con A Induced Supernatants

Four different batches of P and R supernatant were obtained from non-sensitized control spleen cells stimulated with the mitogen con A. Each batch was assessed for its effects on peritoneal cell migration, and in addition, was further investigated as outlined below:

i) The effect of sugar supplementation: Sugar supplementation was used on one batch to determine whether the con A-induced migration inhibition could be reduced, as in the direct assay, by α - L - fucose. This sugar was added to migration wells containing P supernatant to a final concentration of 0.01 M. The migration of cells in 'P' and in 'P plus α - L - fucose, was compared to migration in 'R' supernatant alone.

ii) Sephadex fractionation: Two different batches of supernatant were fractionated on Sephadex. The migration of PEC was determined in medium supplemented with each of the four sephadex fractions. The % migration inhibition for each fraction of P and R was calculated.

iii) Cross-species Activity: One batch of con A supernatant was compared for its effects on the migration of peritoneal cells from rat, Rana temporaria and Xenopus laevis, and head kidney

cells (rich in macrophages) from carp.

The Glucose Uptake Assay:

It has been suggested that one effect of Mammalian MIF is to cause an increase in macrophage metabolism, (Nathan, Remold and David, 1973). One effect of this, is an increase in glucose uptake. In order to compare the uptake of glucose by peritoneal cells exposed to P and R supernatants, a modification of a method given by Onozaki was used (Onozaki, Haga, Ichikawa, Homma, Muira and Hashimoto, 1981). After 72 hours in culture, the medium from migration assay wells was transferred to tubes, and any cells removed by centrifugation. The glucose content of the medium was determined using a glucose oxidase assay (Dahlgrist 1974). Glucose levels were compared in the medium from P and R supplemented wells, to give a % change in glucose uptake, calculated as % change in glucose uptake =

$$100 - \frac{\text{Mean Glucose level in R}}{\text{Mean Glucose level in P}} \times 100$$

A + ve value indicates an increase in glucose uptake in the P supplemented wells, compared to R. Glucose uptake was only determined for one of the fractionated con A supernatants.

Results:

Fig.3.1 illustrates the chronological development of the splenic RFC and serum haemagglutinating antibody titres, following sensitization with either SRBC or FSRBC. Elevated RFC levels were seen 7 days post-immunization, for both antigens, and by 21 days there was a ten fold increase over background levels. FSRBC immunization gave higher RFC numbers than SRBC, although background levels were also higher. Increased haemagglutinating antibody titres were also detected following immunization. Anti-SRBC activity was first apparent at 14 days, with a mean haemagglutination titre of 1.8. This rose to a titre of 4.4 at 21 days post injection. FSRBC animals showed only a low mean titre of 0.8 at 21 days after immunization. This result is in accordance with previous findings for reptiles, where formalinization of erythrocytes reduced their ability to induce a humoral immune response (Baskar and Muthukkaruppan, 1980).

a) The Direct Assay:

Fig.3.2 shows the effect of challenging HGG-sensitized PEC with HGG or HSA, and FSRBC-sensitized PEC with either FSRBC or FRRBC. In both cases the in vitro migration of sensitized cells was significantly inhibited by approximately 40% ($p < 0.05$) following challenge with the sensitizing-antigen. The non-sensitizing antigens had a minimal effect on migration (inhibition of less than 10%) which was not significantly different from the migration seen in normal culture medium ($p > 0.1$). The antigens employed here did not significantly affect the migration of non-sensitized cells (these results are given as the time 0 migration indices in Fig.3.4) Fig.3.3 shows two migration wells, containing HGG-sensitized PEC challenged with either HGG or HSA. Migration was inhibited in the presence of HGG.

The chronological development of the migration inhibition response, following immunization with different antigen preparations, is also given in Fig.3.4. From 7 - 21 days post injection with HGG and FSRBC, antigen specific migration inhibition of greater than 20% was observed ($p<0.05$). A lower inhibition of approximately 20% was also detected at 7 - 21 days following 10% SRBC injections ($p<0.1$) 0.01% SRBC immunization did not give a significant level of antigen induced migration inhibition at either 14 or 21 days after injection.

At 4 days after injection, both HGG- and FSRBC-sensitized cells challenged with specific antigen, gave a significantly increased migration, when compared with control cells ($p<0.05$). This effect was also observed at 5 days for HGG-sensitized cells. FSRBC-sensitized cells, in contrast, displayed a 25% inhibition of migration at this time.

Fig.3.5 demonstrates that the extent of migration inhibition induced by antigen also varied with time in culture. The results presented are mean migration inhibition values for ten different HGG and FSRBC experiments. Antigen-specific migration inhibition could be detected in many cultures by 1 hour, but the small areas of the cell fans at that time, and the variability observed, meant that the inhibition could not be deemed significant. At 18 hours migration inhibition was maximal (mean 28% $p<0.05$) but levels had fallen to 13% by 42 hours and remained low (15%) at 66 hours. In some experiments the 18 hour inhibition was completely lost by 42 hours.

The effect of FCS supplementation on the antigen-induced migration inhibition response are shown in Fig.3.6. The levels of migration inhibition remained the same, for both HGG and FSRBC

challenge, whether FCS was present in the medium or not.

Attempts to modulate the response, by the addition of sugars to the incubation medium are summarized in Fig.3.7. The antigen-induced migration inhibition of both HGG- and FSRBC-sensitized PEC was completely abrogated in the presence of 0.01M α - L - fucose. In contrast, 0.01M β - D - galactose had no such effect on HGG-sensitized cells, but caused a partial reduction in inhibition of FSRBC-sensitized cells. Neither sugar significantly affected the migration of control PEC (α - L - fucose causing a 4% and β- D - galactose a 6% inhibition of migration). A similar effect of α - L - fucose on the HGG-induced migration inhibition of Rana pipiens PEC has also been seen (Gearing, unpublished)

The results of mitogen stimulation on PEC migration are given in Fig.3.8. The T cell mitogens con A and PHA both cause a large migration inhibition, which was reduced by α - L - fucose. This reduction was significant ($p < 0.05$) but the migration inhibition remaining was still high, 45 - 50% and significant ($p < 0.05$) LPS at 200 μg ml^{-1} had no significant effect, although combined with α - L - fucose a stimulation of migration was apparent. At 2000μg ml^{-1}LPS, a 36% migration inhibition ($p < 0.05$) was seen.

b) The Indirect Assay:

Fig. 3.9 summarizes the results of indirect assays, in which the effects of antigen-induced P and R supernatants on cell migration were compared. A decrease in migration of control PEC could be observed in P supernatants when compared to that seen in R supernatants. Significant inhibition was detected in supernatants from 14 days sensitized spleen, and by 21 days, migration indices of up to 66% could be

seen for SRBC-induced supernatant. Although different antigens were used in experiments described here, there does appear to be a trend to increasing migration inhibition activity in spleen culture supernatants with time after injection. No haemagglutinating antibody titres to SRBC could be detected in any of the RBC induced supernatants.

Con A stimulation of spleen cells resulted in the appearance of migration inhibition activity in the P supernatant. The results for one batch of supernatant are given in Fig.3.10. The P supernatant caused a 50% inhibition of migration when compared to R. This inhibition was reduced to 26% when α-L - fucose was added to the migration wells containing P supernatant.

Attempts to isolate supernatant fractions containing the con A-induced migration inhibitory activity are summarized in Fig. 3.11. Two different con A supernatants were investigated, and these gave slightly different profiles of separation. Supernatant A showed significant migration inhibitory activity in fractions I, II and IV with a slight stimulation in fraction III, whilst supernatant B gave significant inhibition in fractions I and III with a lower inhibition in fraction II and very slight stimulation in fraction IV. Supernatant A when assayed before fractionation gave a migration index of 28%. When combined, the overall effect is of a significant ($p < 0.05$) migration inhibition in fractions I and II (with the peak in II) and lower non-significant inhibitions in fractions III and IV. These results are fully discussed in the next section.

Fig.3.12 shows the results of the glucose-uptake assay performed on culture medium taken from the migration wells of fractionated supernatant B. The results are expressed as % change in glucose uptake.

The positive values obtained in fractions II and III indicating an increase in glucose uptake from the medium in P supernatant fractions. Peak uptake was in fraction II which coincided with peak of migration inhibition activity. A contrasting reduction in glucose uptake was observed in fractions I and IV.

Finally, the effect of another batch of con A supernatant on the migration of lymphoid cells from other spe cies is described in Fig.3.13. This supernatant had a migration inhibitory effect of 39% on Rana PEC. The same supernatant when assayed on Rat and Xenopus PEC and carp head kidney cells, gave migration inhibitions of 25%, 15%, and 11% respectively. Of these results, only those for Rana and Rat were significant ($p < 0.05$).

Discussion:

The first series of experiments in this chapter have utilized the direct migration inhibition assay to investigate the antigen specific migration inhibition response of PEC. Following *in vivo* sensitization with protein (HGG) or cellular (FSRBC) antigens, the migration of PEC was reduced after *in vitro* challenge with the specific sensitizing antigens. These antigens had no effect on the migration of non-sensitized cells. This confirms previous findings that a migration inhibition reaction can be induced in Amphibia (Drossler and Ambrosius, 1972; Tahan and Jurd, 1979) and further demonstrates that this reaction is an antigen-specific one, as has been shown for reptiles and fishes see Table 3.1).

The kinetics of the migration inhibition response *in vitro* reveal its transitory nature. Thus, after one hour of culture with the specific sensitizing antigen, sensitized cells may be considerably inhibited in their migration, although some variation is seen at this time. In mammalian and avian systems, inhibition of migration is not detected before six hours in culture (Al Askari and Lawrence, 1972; Zwilling, Barret and Breitenbach, 1972). If the results presented here reflect MIF activity, then this may implicate the prestorage of MIF, or a precursor, which could be released a short time after antigen challenge. The advantages of this to an ectothermic animal, when environmental temperatures may limit synthetic reactions, are obvious. Experiments to investigate the requirements for protein synthesis using the metabolic inhibitor cycloheximide were inconclusive as cycloheximide was found to inhibit the migration of control cells. However, the migration of antigen-sensitized cells challenged with antigen and cycloheximide was inhibited to a greater extent than that of control cells challenged in the same way.

This implies that protein synthesis may not be a prerequisite for MIF production in Amphibia. This would contrast with mammalian results, where cycloheximide and other inhibitors of protein synthesis have been shown to completely abrogate the production of MIF (Varesio, Holden and Taramelli, 1980).

Migration inhibition was found to be maximal at 18 hours but had declined by 42 and 66 hours, in some cases the migration inhibition being completely lost. Similar results have been obtained in mammalian MIF assays (Mooney and Waksman, 1970). This reduction in inhibition has been shown to be a result of the production of lymphokines which stimulate the migration of macrophages. These have been termed either migration stimulation factor or MStF (Aaskov and Anthony, 1976; Kuhner and David, 1976), or macrophage enhancement factor or MEF (Weisbart, Bluestone, Goldberg and Pearson, 1974). The existence of factors with opposing activities such as MIF and MEF, and their sequential production (MIF being produced over the first 24 - 48 hours, followed by MStF from 48 - 120 hours) means that the time at which migration inhibition is measured following exposure to antigen in vitro is of great significance. It must be noted that amongst the reports of migration inhibition, reactions in lower vertebrates, only those of Drossler and Ambrosius (Drossler and Ambrosius, 1972) on Bufo bufo, and Jayaraman and Muthukkaruppan (Jayaraman and Muthukkaruppan, 1977) on Calotes versicolar, have investigated migration inhibition values at times later than 24 hours of culture. In both cases, migration inhibition values were found to be unchanged at 48 hours of incubation, compared to the 24 hour values. This may reflect either different kinetics of MIF and MEF production in these species, or that the inhibition observed was due to non-lymphokine factors, such as antigen/antibody complexes. This issue remains to be resolved.

Further evidence for the existence of both migration inhibitory and stimulatory factors has emerged from the study of the time course of sensitization. Thus, following sensitization with HGG, significant migration inhibition ($p < 0.05$) was detected from 7 to 21 days post immunization. Sensitization with 10% SRBC gave a lower degree of migration inhibition than did HGG-sensitization over the 7 - 21 days period. However, sensitization with 10% FSRBC raised the migration inhibition observed to levels seen for HGG. Treatment with a low dose of SRBC could not induce a significant migration inhibition response to the sensitizing antigen. These results are similar to those found in reptiles (Baskar and Muthukkaruppan, 1980) and teleost fish (Jayaraman, Mohan and Muthukkaruppan, 1979) which showed that chemical modification of erythrocyte antigens, by formalin or periodate oxidation, leads to an increase in cell mediated immune reactions, including the migration and inhibition response, with an accompanying decrease in humoral reactivity, such as serum antibody titres and plaque forming cell (PFC) responses. Dennert and Tucker (Dennert and Tucker, 1972) have suggested that formalinization makes SRBC resistant to macrophage phagocytosis and processing, leading to an effective decrease in the dose of antigenic determinants presented to lymphocytes. The migration inhibition response has been shown to be inversel y related to the sensitizing dose of antigen (MacKaness, Lagrange, and Ishibashi, 1974) whereas PFC levels are directly related to antigen dose (Jayaraman, Mohan, and Muthukkaruppan, 1979). The results presented in this chapter would appear to be consistent with this proposed relationship, since FSRBC sensitization gave a greater degree of migration inhibition but lower serum antibody titres, than SRBC sensitization.

The present results however, do not demonstrate an inverse relationship between migration inhibition and the sensitizing dose of SRBC. The lack of response following 0.01% SRBC injection may be a

consequence of intraperitoneal immunization. The peritoneal exudate population includes a high level of phagocytic PMN leucocytes which will be in competition with peritoneal macrophages and monocytes for phagocytosis of the antigen. It may be that this competition prevents sufficient antigen being taken up by macrophages to enable them to prevent an immunogenic dose of antigen to the T lymphocytes, and therefore sensitization is not achieved. This possibility is made more likely when it is considered that not all peritoneal macrophages are involved in antigen presentation to lymphocytes. Thus, in mice two distinct populations of peritoneal macrophages are observed, one which carries MHC I region antigens and which processes antigen for presentation to lymphocytes, and another which does not carry I region antigens and is highly phagocytic, but does not interact with lymphocytes (Tzehoval, De Baetselier, Feldman and Segal 1981). It may be that if the low dose of SRBC given here had been introduced by another route, for example intravenously, then the predicted high levels of migration inhibition seen in mice would have been detected.

The increased migration of sensitized PEC seen here following specific antigen challenge at 4 - 5 days for HGG and 4 days for FSRBC sensitized cells, contrasts with the 7 - 21 days results obtained for these antigens. It also differs from results obtained by Jayaraman and Muthukkaruppan following allografting of lizards (Jayaraman and Muthukkarappan 1977). Their results demonstrated antigen specific migration inhibition at 4 days after grafting to alloantigens. The results presented here may again be a consequence of the route of sensitization as the migrating cell population is being removed from the same site as the exudate inducing and immunizing injections. Poulter and Turk have shown that macrophages can be made unresponsive to MIF by giving a high

de-sensitizing dose of antigen (Poulter and Turk, 1976). They have suggested that this unresponsiveness is due to the macrophages becoming activated, and have observed that it persists for 3 - 7 days. It may be that a combined dose of thioglycollate and antigen, given for the 4 and 5 day assays, is causing activation of the peri toneal cells and thus making them unresponsive to MIF. This would leave them open to the effects of migration stimulatory factors, which in mammals are known to be produced 2 - 5 days after antigenic challenge, and are released by lymphocytes responding to the sensitizing antigen (Weisbart, Bluestone, Goldberg and Pearson, 1974). Any MIF produced by the in vitro challenge with sensitizing antigen would thus have no effect on macrophages rendered un-responsive as a result of the combined injections.

It has been suggested that migration inhibition activity may be generated by the action of enzymes or components of foetal calf serum, or that MIF may require the presence of foetal calf serum for its action. The work of Fox and co-workers, has also demonstrated that FCS itself contains both migration inhibitory and stimulatory factors similar in properties to the equivalent lymphokines (Fox, Gregory and Feldman 1974; Fox, MacSween and Rajaraman, 1978). It is therefore encouraging in the search for amphibian lymphokine MIF to find that antigen specific migration inhibition occurred independently of foetal calf serum supplementation.

It is perhaps pertinent, at this point, to restate that the migrating peritoneal cell population is a heterogeneous one, mainly comprising monocyte/macrophage and polymorphonuclear leucocyte cells, as judged by histological examination of Leishmann's stained smears. Thus,it is not certain whether macrophage MIF or PMN-MIF, or both, are being assayed in this system.

Where stimulation of migration is concerned, however, no stimulatory lymphokines for PMN leucocytes have been described in mammalian systems, and it may be reasonable to assume that the monocyte/macrophage cell line is being affected.

A characteristic feature of both mammalian MIF and PMN/MIF, is that their action can be blocked by the presence of certain sugars in the incubation medium. Thus, MIF has been shown to be blocked by α- L - fucose (Remold 1973) as well as by other five methyl pentose sugars (Rocklin 1976,b) and PMN-MIF could also be blocked by α - L - fucose (Bendtzen, 1975), or by N-acetyl-D-galactosamine (Rocklin 1976,b) These results for the direct assay indicate that for Rana temporaria (Gearing, unpublished observations) (and also Rana pipiens)/the antigen specific migration inhibition response, to both HGG and FSRBC, can be blocked by α- L-fucose but not β - D - galactose. Although only two sugars were tested, it is significant that mammalian and amphibian migration inhibition reactions are blocked by the same sugars. These findings are consistent with the idea that α- L - fucose competes with a cell surface receptor which contains α - L - fucose as an essential terminal sugar as part of its binding site for MIF (see Figure 3.14).

The results obtained here/with the direct assay were extended by employing mitogen stimulation, with both T and B cell mitogens. Such studies had been prepared on the Gar Lepisosteus platyrhinus by McKinney and co-workers (McKinney Ortiz, Lee, Sigel, Lopez, Epstein and McLeod 1976). They added mitogens to the incubator medium of a direct assay, and observed migration inhibition with PWM, CON A and PHA. The extent of the inhibition varied depending on the lymphoid tissue used. Interpretation of their results is, however, complicated by the fact that most mitogens are also potent agglutinating agents, and the observed inhibition may merely reflect the agglutination of the migrating cells by the mitogens.

Experiments described in this chapter which employ mitogens in direct migration inhibition assays confirm McKinney's results, but their significance is increased by the use of the sugar inhibition technique. Thus, both con A and PHA caused a highly significant degree of migration inhibition when given at the optimally mitogenic doses of 50 μg PHA ml^{-1} and 1 μg con A ml^{-1}, but this inhibition was significantly reduced ($p < 0.05$) by 0.1 M ∝ - L - fucose, although a significant inhibition of migration still remains. The residual migration inhibition could be due to either an agglutinating effect, or to MIF being produced in high enough amounts to overcome the sugar blockade, or to a combination of both. An agglutinating component for con A is implicated by the increased migration inhibition induced by the high, but suboptimal mitogenic dose of CON A (10μ g ml^{-1}). The mitogens used, have no intrinsic affinity for ∝ - L - fucose, and hence any reduction in migration inhibition observed was not due to failure of mitogen binding to its target cells.

Using LPS, a B cell mitogen, at 200 u g ml^{-1}, did not significantly affect migration, although the addition of ∝ - L - fucose reversed the slight inhibition to a stimulation of migration. This may reflect a low level of MIF production by LPS stimulated B cells as indicated by Rocklin and co-workers (Rocklin, MacDermott, Chess, Schlossman and David 1974), or result from a potentiating effect of LPS on the background MIF in the cultures, as has been demonstrated by Fox and Rajaraman (Fox and Rajaraman 1980). Addition of 2000μ g ml^{-1} LPS did cause a significant inhibition of migration ($P < 0.05$) although by analogy to mammalian results, this may be due to direct effects on the peritoneal macrophages causing the release of a variety of pharmacologically active mediators, including interleukin 1, pyrogens and lyzozymal enzymes (Oppenheim, Mizel and Meltzer 1979).

In fact, recent unpublished studies by the author have demonstrated interleukin 1 production by Xenopus laevis peritoneal cells, following LPS stimulation. Thus, the significance of the migration inhibition results presented here remains doubtful. Work performed in mice has shown that removal of peritoneal T cells by anti Thyl antibodies and complement, also abrogates the migration inhibition response, and here implies that the T cells are the source of peritoneal MIF (Clinton, Magoc and Aspinall 1974). Again the significance of these findings to Amphibian cells is not resolved.

Although the direct assay results go a long way towards establishing the existence of lymphokines affecting cell migration, they cannot preclude a direct cell-cell contact mechanism causing all of the observed effects. To confirm the existence of soluble mediators required the use of an indirect assay. Results obtained from these experi ments demonstrated that antigen challenge of sensitized splenocytes in vitro caused the release of soluble material which inhibited the migration of non sensitized cells. This inhibition of migration caused by P compared to R supernatant is in agreement with mammalian (Bloom and Bennet 1966) and avian work (Zwilling, Barret and Breitenbach 1972).

The trend to increasing MIF activity, in antigen induced supernatants with time after immunization, lags behind that detected by the direct assay. Significant levels of migration inhibition were only observed from 14 days onwards. This delay may be caused by the dilution of the supernatants which was necessary in order to fill sufficient replicate migration wells, and not by a lack of MIF production by spleen cells, 7 days post immunization. Manning has reported, however, that for Xenopus laevis, 14 days is required before detectable amounts of antigen localize in the spleen,

following an intraperitoneal injection (Manning, 1981). The Direct assay may then be expected to detect the in situ response to an antigen injected intraperitoneally, at an earlier time than the indirect assay.

Mitogen stimulation of spleen cells by con A also gave rise to the production of migration inhibition activity, similar to that demonstrated in mammalian species (Pick, Brostoff, Krejci and Turk, 1970) and also recently, by the author in Xenopus laevis (Gearing, unpublished). In Rana the migration inhibition ativity induced by con A, was also shown to be blocked by α - L - fucose. Thus the migration inhibition reaction induced in Rana temporaria by specific antigen or mitogen, would seem to be mediated by soluble material whose effect is blocked by α - L - fucose. The involvement of antibody in the migration inhibition response would appear to be precluded by the blocking effects of fucose, and by the lack of a detectable haemagglutinating antibody titre in any of the SRBC-induced supernatants tested.

Sephadex fractionation studies were employed here in an attempt to throw more light on the nature of themediator(s) involved. Although slightly different profiles of inhibitory activity were seen in the two batches of supernatant, the nett effect demonstrates a broad range of inhibition in fractions from 9 - 100,000 Daltons, with a maximal inhibition in the 27 - 50,000 Dalton MW range. Very similar results were found in early attempts to purify guinea pigs MIF (Dumonde, Page, Matthew and Wolstencroft, 1972). Recent work by various groups has found MIF activity associated with a variety of MW ranging from 15,000, 30,000, 45,000, 50,000, 60,000 and 86,000 Daltons in guinea pigs; 12 - 100,000 and 48 - 67,000 Daltons for mouse; and 23 - 43,000 and 65,000 Daltons for humans (Yoshida, 1979 ; Sorg and Klinkert, 1978; Kuhner and David, 1976; Altboum and Pick, 1979; Weiser, Greineder, Remold and David, 1981). Sorg and Klinkert have

proposed that this diversity of MW reflects a sub unit structure for MIF. They identified a 15,000 Dalton MIF which can associate with other similar molecules to yield 30,000, 45,000 and 60,000 Dalton MIF. (Sorg and Klinkert, 1978).

The conclusions that may be drawn about Rana MIF from these separation results, must be tentative, pending further investigation. However, the broad range of activity would seem to implicate more than one discrete MIF molecule in the P supernatants. Examination of the inhibition profiles for the two different batches of supernatant reveals both a high and a low MW peak of MIF activity. The two peaks are separated by a fraction showing either low inhibition or slight stimulation. In supernatant A, the two peak activities are in fractions I and III, and in supernatant B they are in fractions II and IV. Assuming some variation in column performance has affected separation in the two runs, it could be postulated that at least two MIF molecules, with MW at the borderlines between fractions I and II, and III and IV respectively, are being studied. These two MIF molecules would have MW of approximately 50,000 and 16,000 Daltons, other intermediate MW MIF cannot however be excluded. More sophisticated separation procedures would serve to confirm or refute this speculation.

Other workers have shown a lack of species specificity for mammalian MIF (Pick 1979), and this is further confirmed for Rana MIF, which is even active on rat peritoneal cells. The phylogenetic conservation of amphibian and mammalian MIF sugar inhibition properties, may suggest a fundamental role for MIF in the vertebrate immune system. Recently, it has been suggested that the in vivo significance of MIF molecules lies not only in their effects on the migration of phagocytes, but also in other immunological activities. Thus mammalian MIF cannot be physicochemically or immunologically separated from another mediator, macrophage activity factor or MAF (Onozaki, Haga, Ichikawa,

Homma, Miura and Hashimito, 1981) MAF activity is manifested in a variety of ways, including enhanced adherence, phagocytosis, pinocytosis, bacteriostasis and tumoricidal activity, it is also accompanied by the increase in activity of certain enzyme systems, including the oxidation of glucose through the hexose monophosphate shunt (Reviewed in Nathan Remold and David, 1973). These MAF effects are also paralleled to some extent by similar effects on PMN-MIF on PMN leucocytes (Rocklin, 1978). Such in vitro activation has an in vivo counterpart in the enhanced function of macrophages observed in animals infected with certain mico-organisms (Karnovsky and Lazdins, 1978).

An activating function for Rana MIF is supported by the effects of fractionated supernatant on the glucose levels in migration wells. Peak glucose uptake was achieved by peritoneal cells incubated in medium supplemented with the fraction of supernatant containing peak MIF activity. Further experiments are required to confirm this relationship for Rana MIF and MAF.

The role of MIF in endotherm immunity is further confused since, in mammals, it cannot be physicochemically separated from soluble immune response suppressor or SIRS (Tadakuma, Kuhner, Rich, David and Pierce, 1976) a lymphokine which ac ts on macrophages, causing them to secrete a factor which suppresses plaque forming cell responses (Aune and Pierce, 1981). The situation is further complicated by the work of Fox and Rajaraman, which demonstrates that in the mouse, MIF is produced by T helper cells and MStF by T suppressor cells, and also that MIF enhances PHA transformation of lymphocytes and MStF suppresses it (Fox and Rajaraman, 1979; Fox and Rajaraman, 1981). The invivo significance of such findings is still unclear, but they add support to the notion that MIF (and MStF) may play a more fundamental role in the immune response than simply

controlling cell migration. The investigation of such helper and suppressor functions in amphibians would be of great interest, and may provide further evidence for the functional division of T cell types implicated by other authors (Horton, Rimmer and Horton, 1977) (Ruben, Mette, Cochran and Edwards, 1980) (Charlemagne, 1979) and by the studies presented in Chapter Five of this thesis.

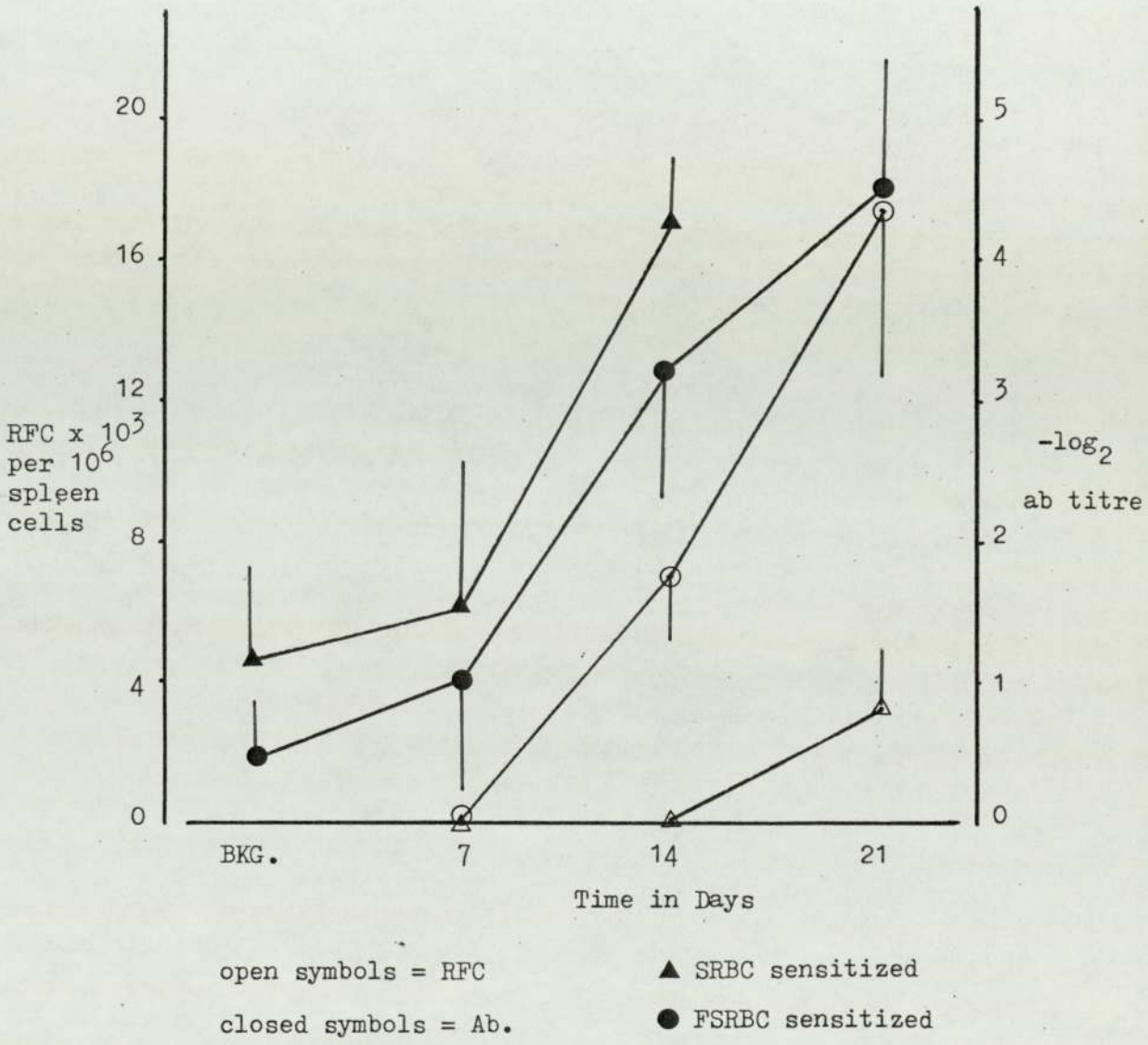


Fig.3.1 Kinetics of splenic RFC production, and serum haemagglutinating antibody titres, following immunization with either SRBC or FSRBC.

Each point is themean value for five different animals. The BKG values are for non-immunized animals. Both RFC and antibody titres were determined using SRBC as the test antigen.

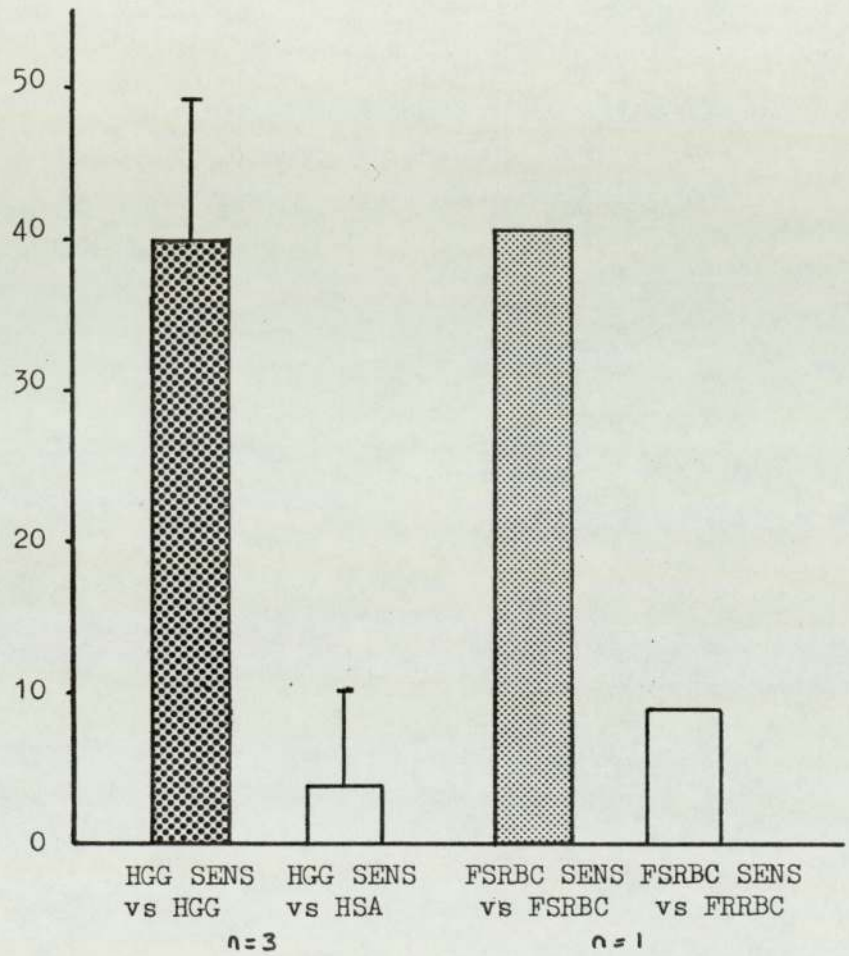


Fig.3.2 The antigen specific, direct migration inhibition response of PEC.

The results compare the inhibition of migration of antigen sensitized PEC challenged in vitro with the sensitizing or a different antigen, 21 days after immunization. Vertical bars represent the standard error of the mean.

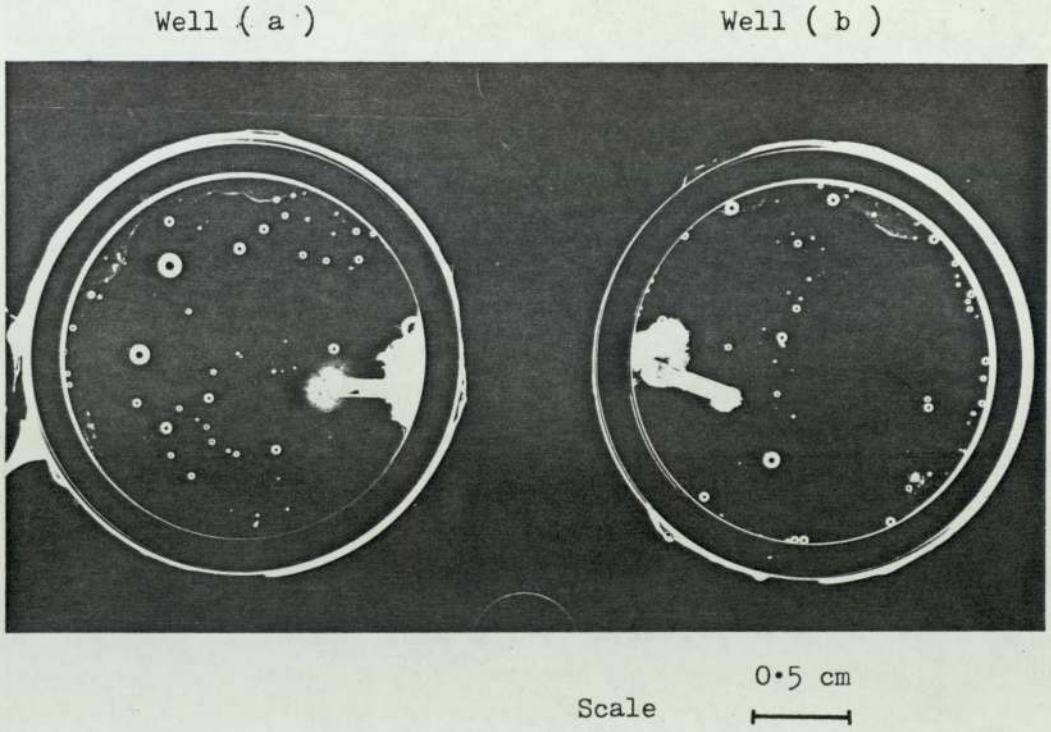


Fig. 3.3 Photograph showing migration of HGG sensitized PEC into wells containing HSA (a) or HGG supplemented (b) medium.

The migration area of the cell fan in (a) was no different from that in culture medium alone. This illustrates the antigen specificity of the direct migration inhibition reaction. PEC were harvested 21 days after *in vivo* sensitization with HGG.

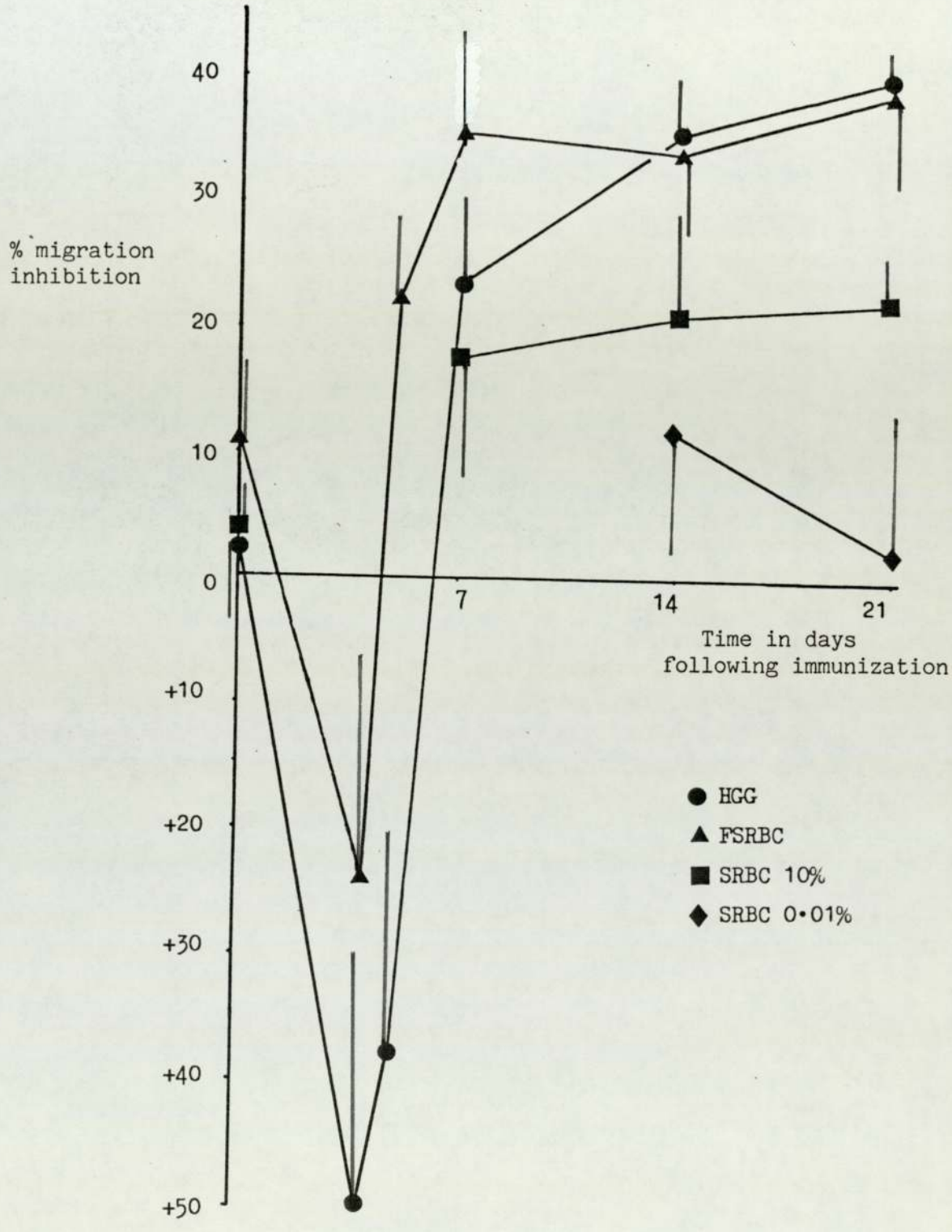


Fig.3.4 Kinetics of the antigen specific direct migration inhibition response following immunization with different antigens.

Each point is the mean of from two to three separate experiments for the antigen induced inhibition of migration, following sensitization with the antigens indicated. Inhibitions of greater than 20% are usually considered significant.

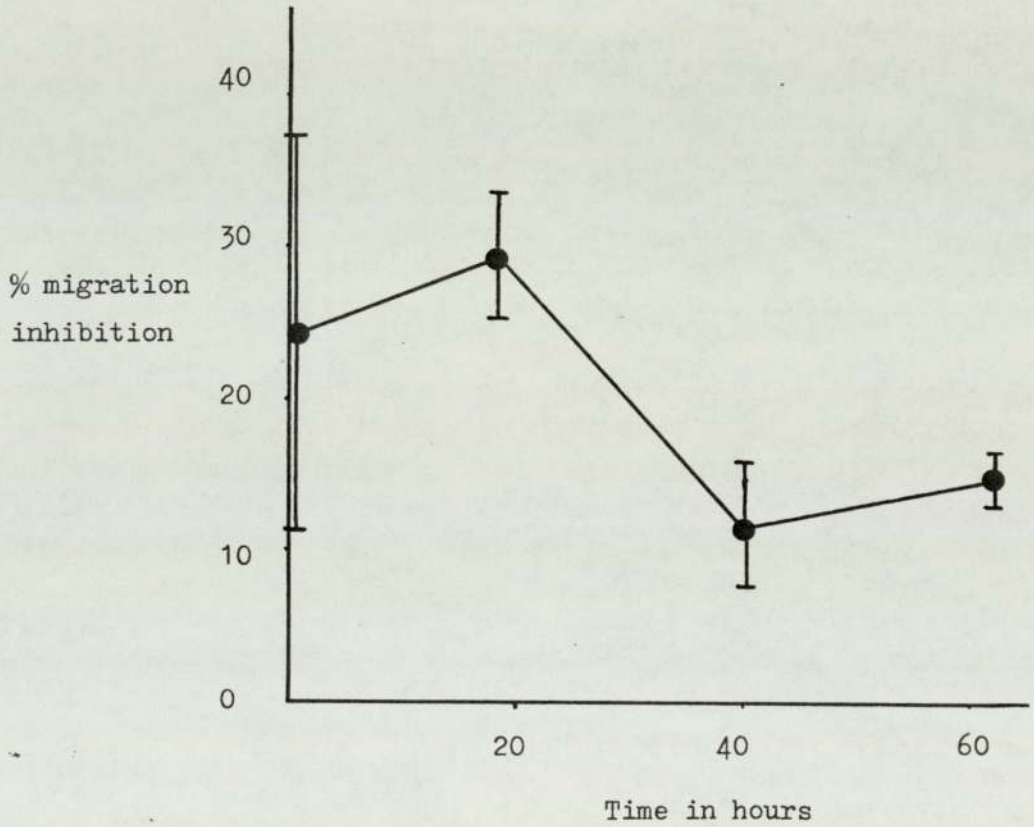


Fig. 3.5 The development of the migration inhibition response with time in culture.

Each point represents the mean migration inhibition value for 10 separate experiments, using a variety of antigens. The vertical bars indicate the standard error of the mean value.

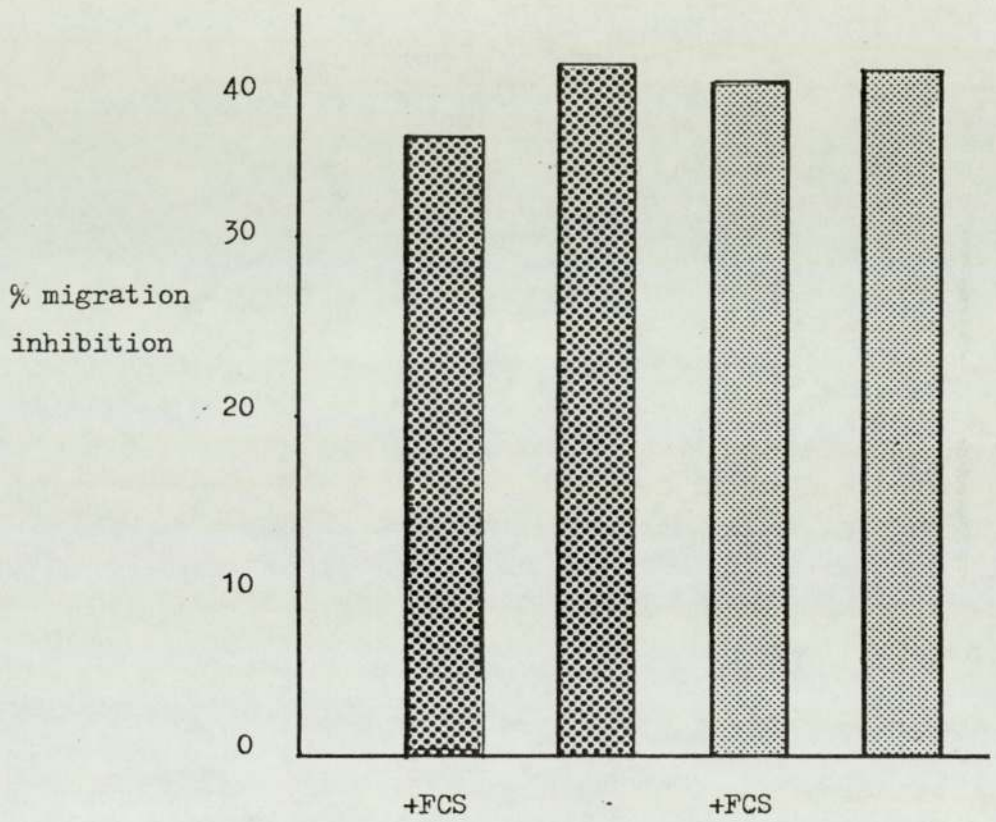


Fig. 3.6 The effect of foetal calf serum supplementation on the antigen specific migration inhibition response.

The dark bars represent HGG , and the light bars FSRBC induced migration inhibition reactions, in culture medium containing 1% FCS or in medium alone. n=1.

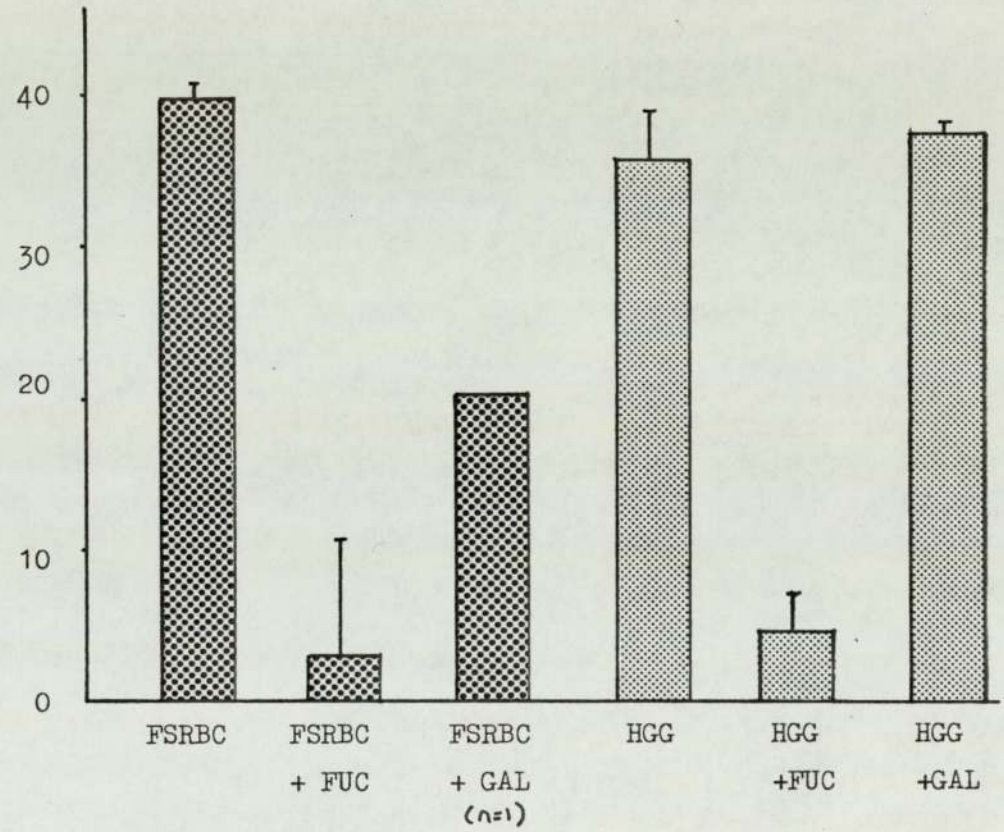


Fig. 3.7 The effect of sugar supplementation on the antigen specific migration inhibition response.

The dark bars represent FSRBC, and the light bars HGG induced migration inhibition reactions. The effects of adding 0.01M α - L - fucose, and 0.01M β - D galactose are shown. Vertical bars indicate the standard error of the mean migration inhibition, $n = 2$.

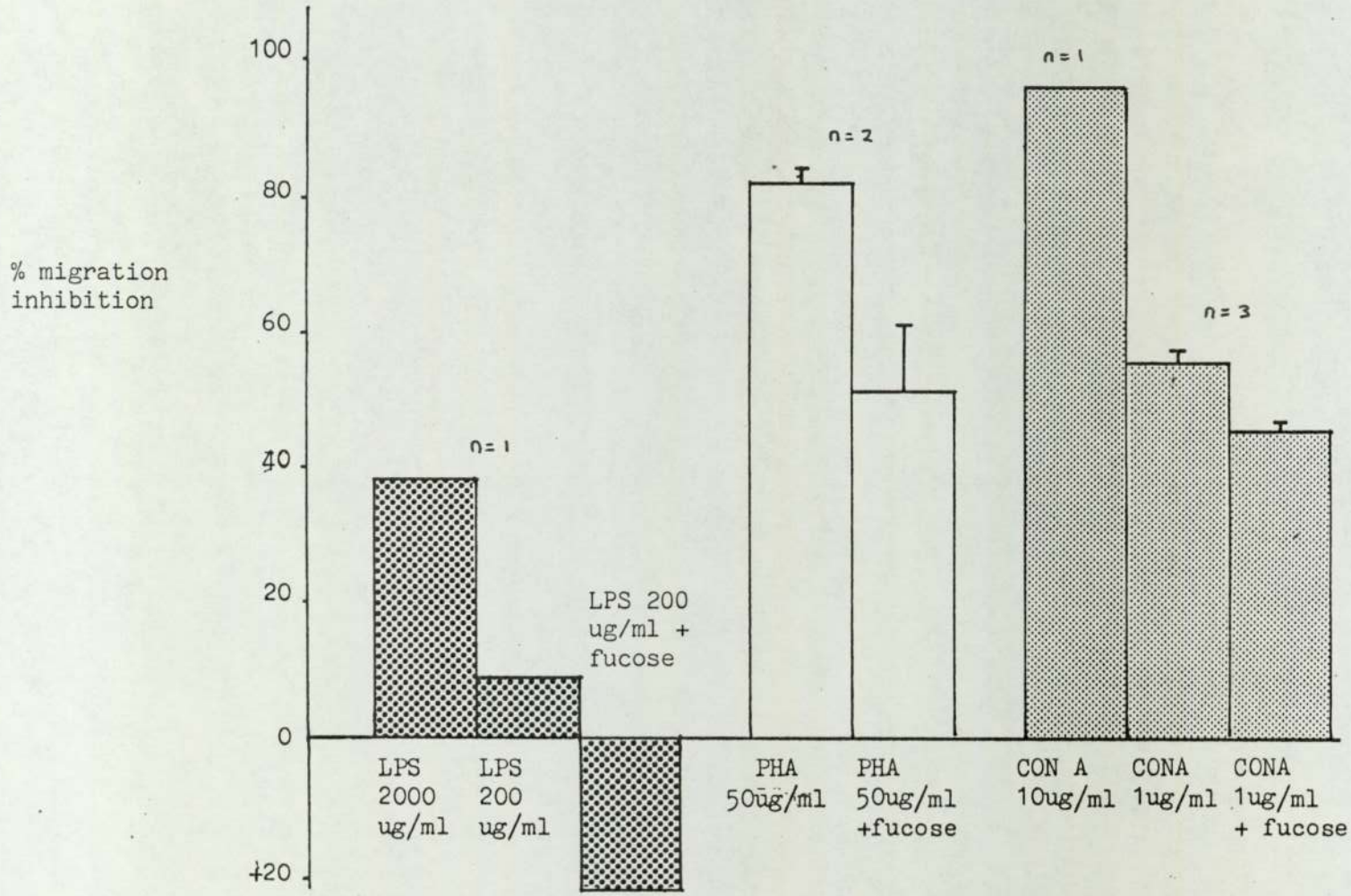


Fig. 3.8 The direct migration inhibition response induced by T and B cell mitogens, and the effect of ∝ - L - fucose.

Migration of control PEC in the presence of mitogens was compared with migration in medium only. In some cases 0.01 M fucose was also included in the mitogen containing wells.

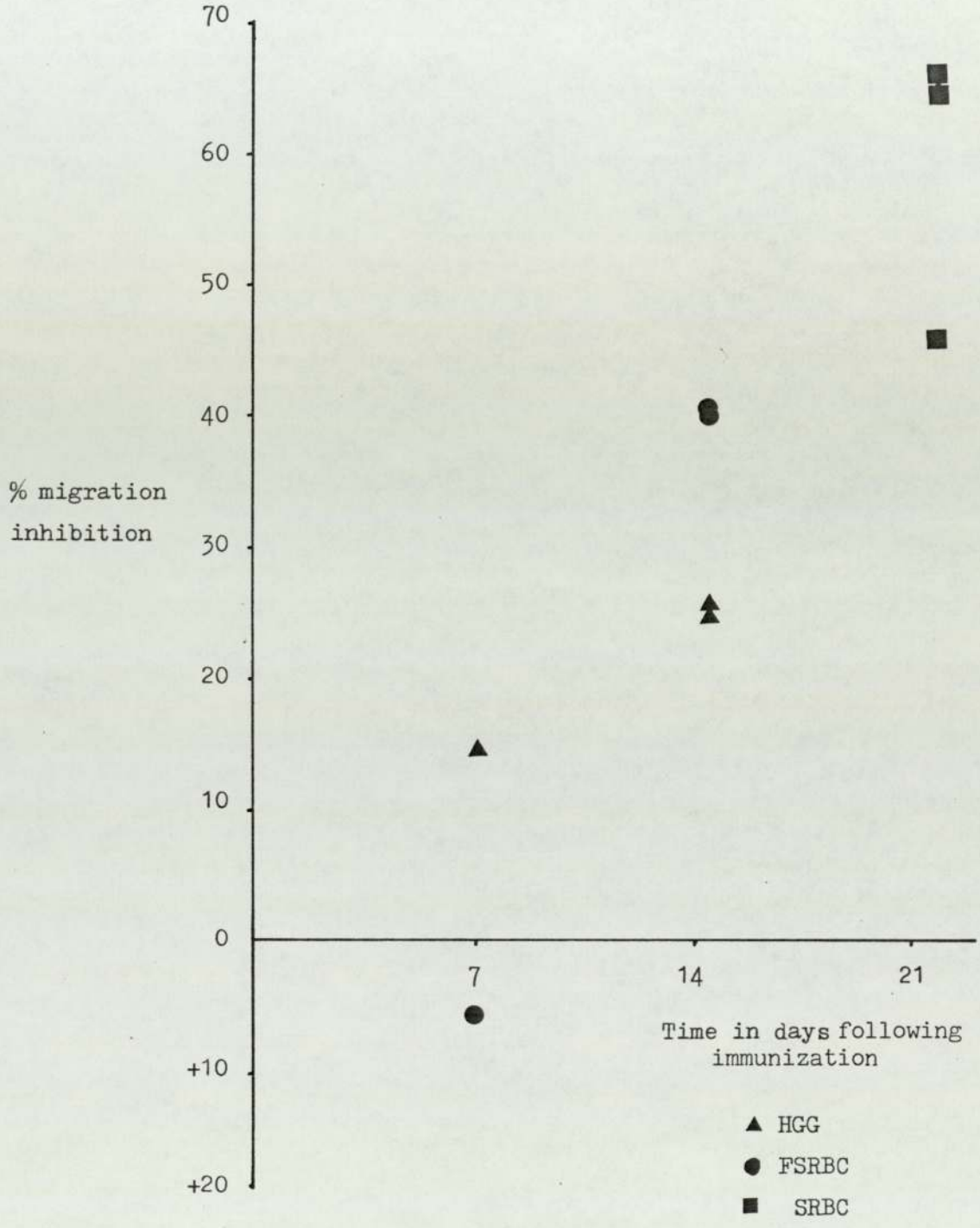


Fig. 3.9 Kinetics of migration inhibitory activity production in specific antigen induced spleen cell culture supernatants.

Each point represents the migration inhibition value, comparing P with R supernatants harvested from spleen cell cultures at different times following immunization.

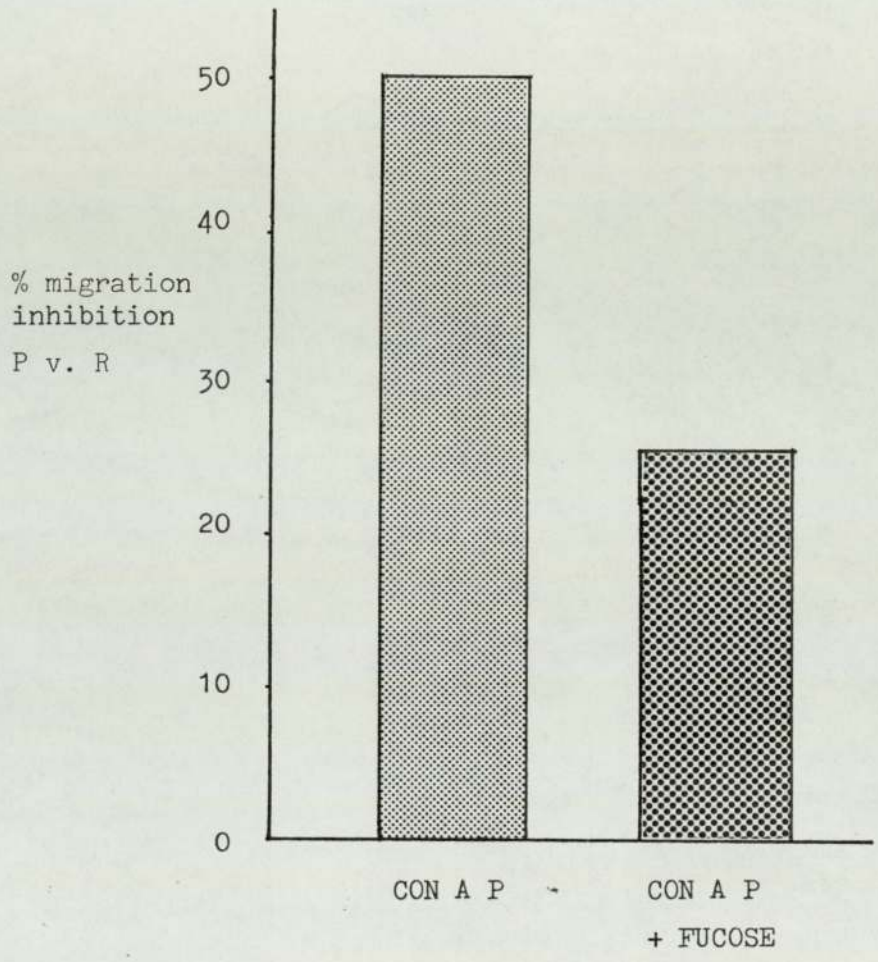


Fig.3.10 Migration inhibitory activity in con A induced spleen cell culture supernatants and the effects of ∝ - L - fucose

The migration inhibition comparing the Con A P with Con A R supernatant is given by the light bar, the dark bar shows the effect of 0.01M ∝ - L - fucose on the P supernatant.

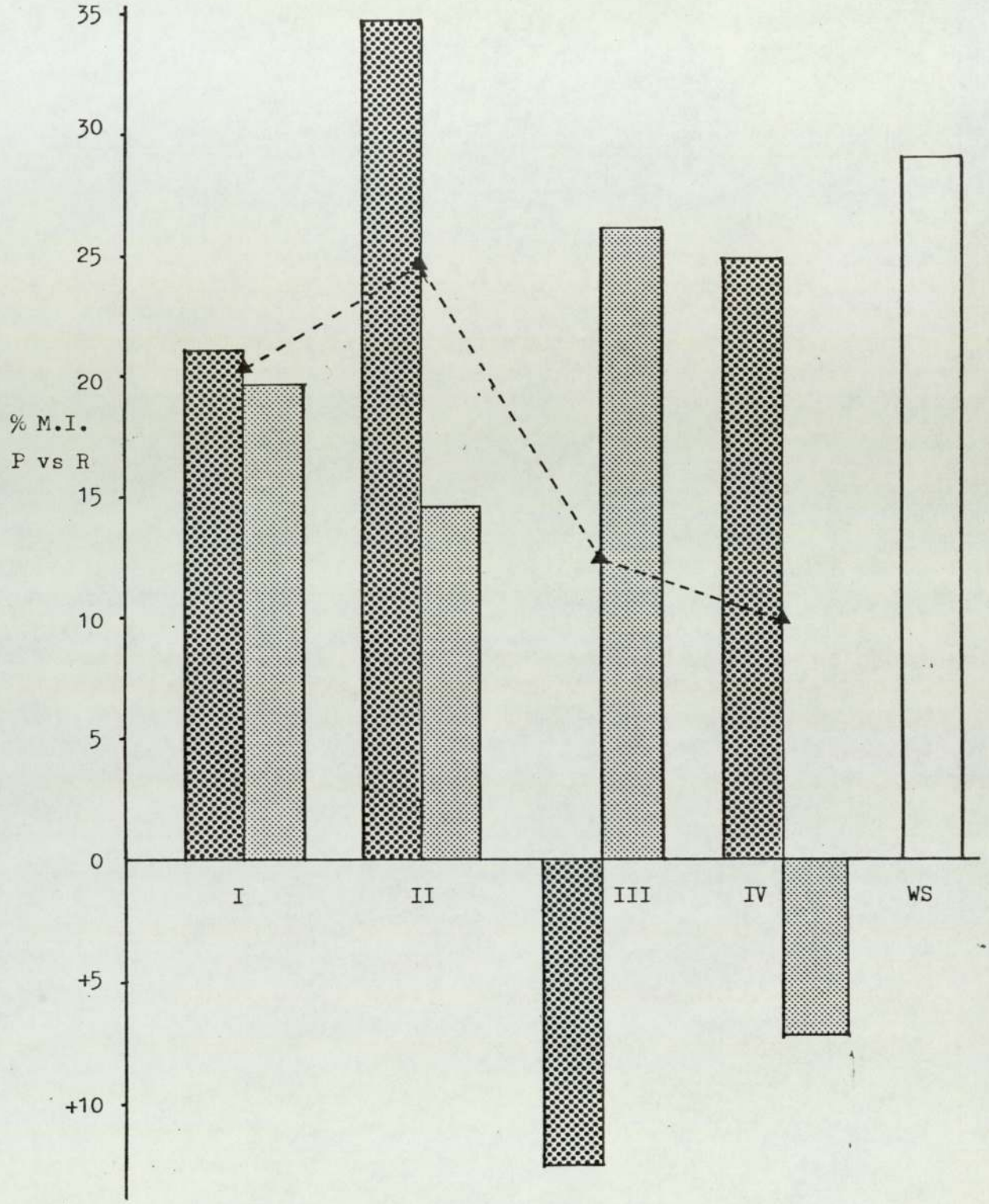


Fig.3.11 Comparison of sephadex fractions of con A induced, P and R, supernatants for their effects on the migration of PEC in the indirect migration inhibition assay.

The bars indicate % MI values, comparing P to R for two different con A induced supernatants. The mean % MI for each fraction is represented by ▲ . The mean % MI for unfractionated supernatants is given in the WS column.

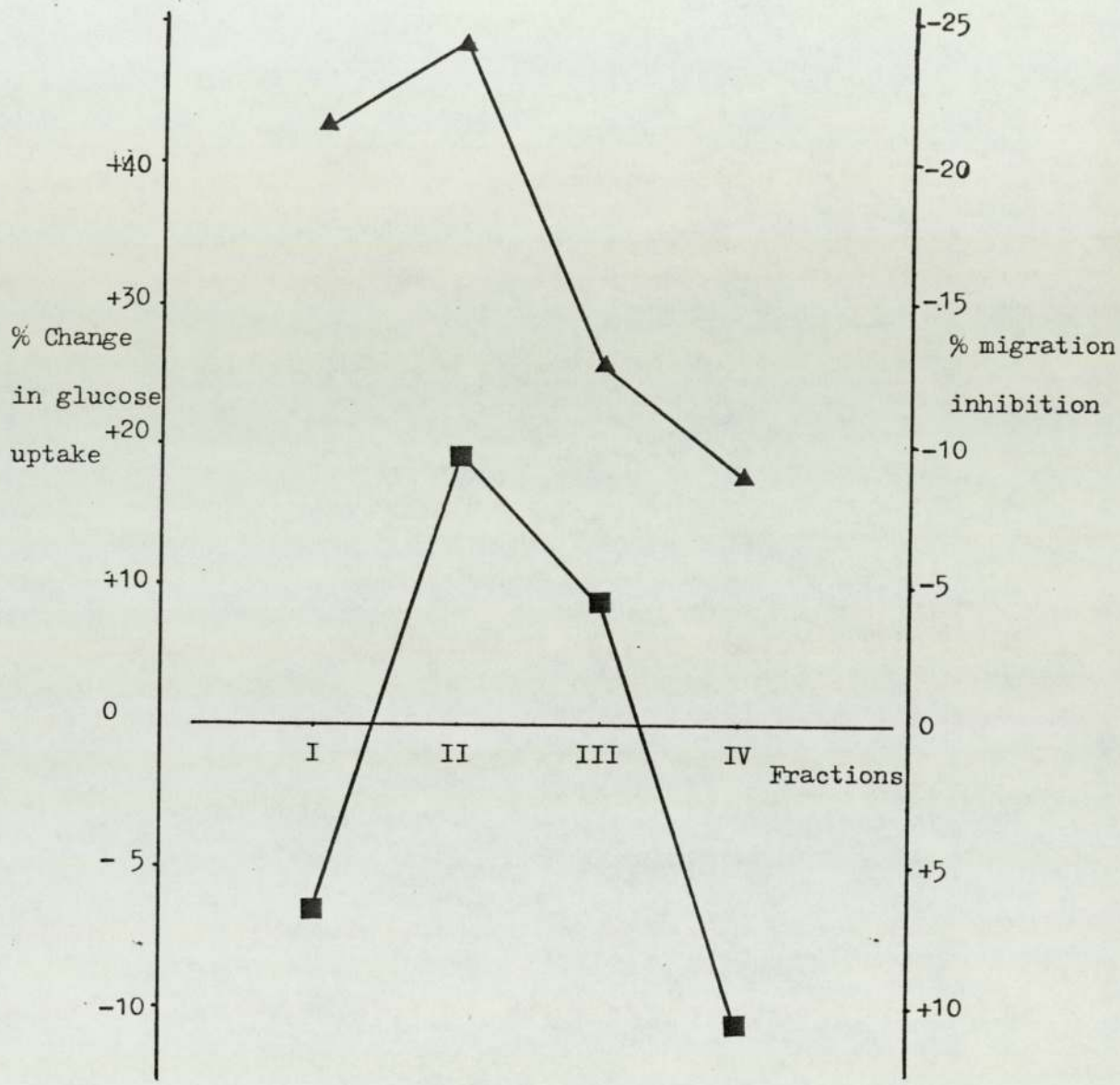


Fig.3.12 Comparison of sephadex fractions of con A induced, P and R, supernatant for their effects on the uptake of glucose by PEC.

Each point ■ represents the % change in glucose levels in the medium of migration wells in which PEC have been cultured for 72 hours. A positive value indicates more glucose uptake by cells cultured in P supernatant fractions than in R. The ▲ symbols summarize the migration inhibition values for each fraction which were given in Fig.3.11.

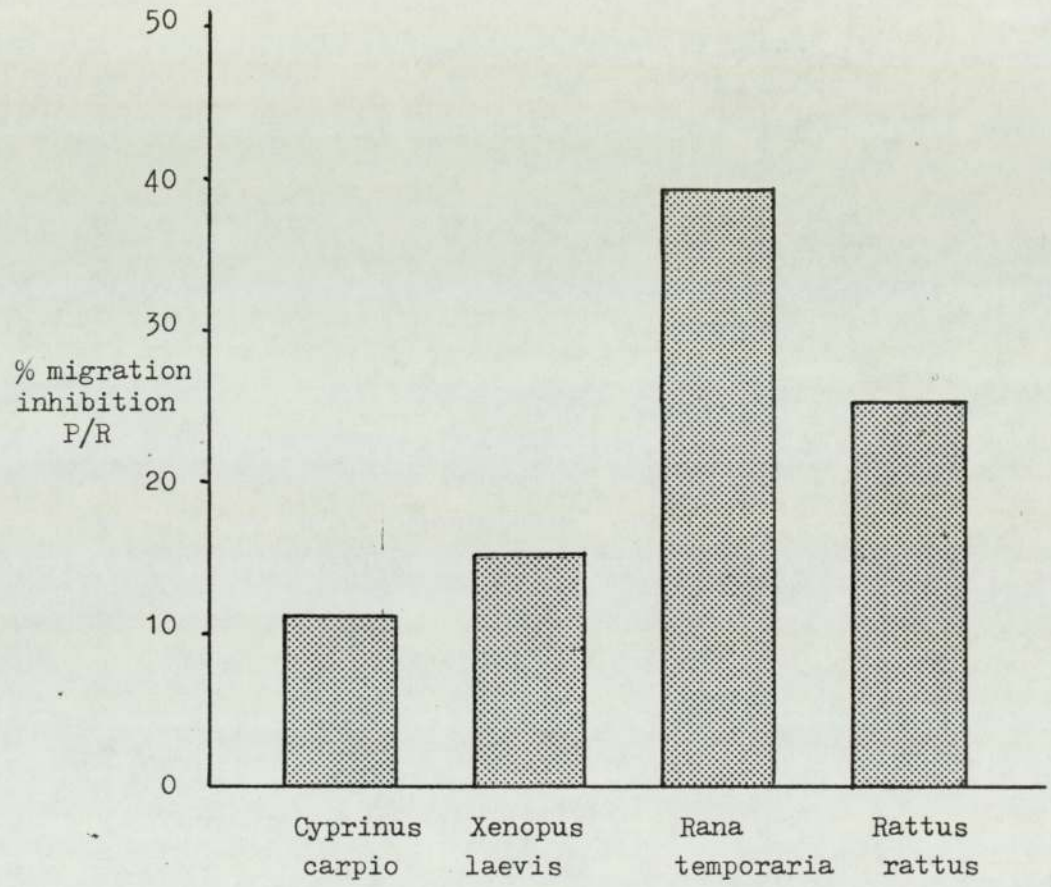


Fig. 3.13 The effect of Rana temporaria, con A,P and R supernatants, on the migration of lymphoid cells from other species. The same batch of con A-induced Rana P and R supernatant, was tested for its effect on the migration of C. carpio head kidney cells, X. laevis PEC, R. temporaria PEC and R. rattus PEC. The % migration inhibition compares the extent of cell migration in P and R supplemented culture medium, using the capillary tube assay. The greater the % inhibition, the more cell migration was inhibited in the P supernatant.

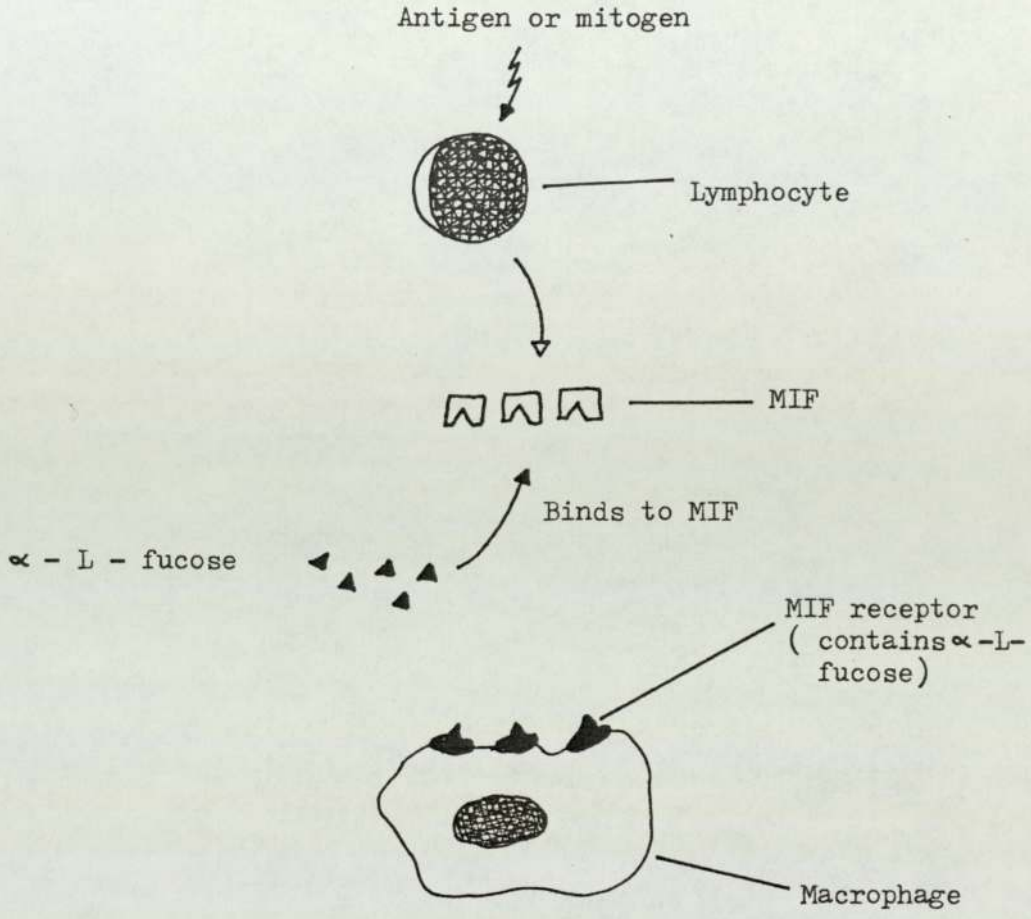


Fig. 3.14 The effect of ∝-L-fucose on the interaction of MIF and the macrophage, in mammals.

Table 3.1 Phylogeny of migration inhibition reactions.

Class	Species	Stimulus	AG Specificity	Nature of Mediator	Ref
BIRD	Chicken	Allograft, soluble and cellular antigens	+	T and B cell derived lymphokines	1.2. 3.4. 5.6.
REPTILE	Calotes versicolor	Allograft. FSRBC SRBC	+	Non Ab[a]	7.8. 9.
AMPHIBIA	Bufo bufo	PPD	NT	NT	10.
	Ambystoma mexicanum	PPD	NT	Non Ab[b]	11.
FISH	Tilapia mossambica	SRBC FSRBC	+	Non Ab[c]	12.
	Lepisosteus platyrhircus	Allograft mitogen	+	NT	13. 14.

a) Migration inhibition to FSRBC in absence of serum ab and PFC in spleen

b) Migration inhibition transferrable by splenocytes not by serum

c) Migration inhibition and PFC responses inversely related

References:

1. (Zwilling, Barret and Breitenbach, 1972)

2. (Morita and Soekawa, 1972)

3. (Fauser, Purchase, Long, Velicer, Mallman, Fanser and Winegar, 1973)

4. (Timms, 1974)

5. (Subba Rao and Glick, 1977)

6. (Stinson, Mashaly and Glick, 1979)

7. (Jayaraman and Muthukkaruppan, 1977)

8. (Jayaraman and Muthukkaruppan, 1978)

9. (Baskar and Muthukkaruopa, 1980)

10. (Drossler and Ambrosius, 1972)

11. (Tahan and Jurd, 1979)

12. (Jayaraman, Mohan and Muthukkaruppan, 1979)

13. (McKinney, Ortiz, Lee, Sigel, Lopez, Epstein and McLeod, 1976)

14. (McKinney, McLeod and Sigel, 1981)

CHAPTER FOUR

STUDIES OF ANTIGEN AND MITOGEN INDUCED CHEMOTACTIC FACTORS

Introduction

The massive localised influx of cells which accompanies delayed type hypersensitivity reactions, allograft rejection, and certain pathogenic and parasitic infections, is a key feature of the immune response. The accumulation of effector and phagocytic cells at sites of antigenic challenge is largely dependent upon a process called chemotaxis, in which the cells involved undergo oriented movement along a chemical gradient. Leucocyte chemotaxis was first described almost a century ago, and the migration, homing and congregation of leucocytes at inflammatory sites is known to be influenced by a variety of stimulating factors (reviewed in Gallin and Quie, 1978 and Klebanov and Clark, 1978).

Chemotactic factors may be of extrinsic (bacterial) or host origin, and the latter group is known to include lymphokines. Stimulation of lymphocytes has been shown to result in the production of soluble factors with chemotactic activity for macrophages (Ward, Remold and David, 1969); polymorphonuclear leucocytes (Ward, Remold and David, 1970) basophils (Kay and Austen, 1972); eosinphils (Cohen and Ward, 1971); and lymphocytes (Ward, Offen and Montgomery, 1971). Lymphocyte - derived chemotactic factors (L.D.C.F.) have been described in a number of mammalian species including man (Altman, Chassy and Madder, 1975) and also in the chicken (Altman and Kirchner, 1974).

Large localized accumulations of leucocytes have been extensively described during the immune response of lower vertebrates,

(including the Amphibia) particularly in association with allograft and xenograft destruction (see Manning and Turner 1976). Recently Tahan and Jurd have also reported mononuclear cell accumulations at sites of experimentally induced cutaneous delayed type hypersensitivity reactions in the urodele amphibian Ambystoma mexicanum (Tahan and Jurd 1979); similar findings were also reported for the rainbow trout Salmo gairdneri (Bartos and Sommer, 1981).

Whilst these in vivo observations are highly suggestive of chemotactic activity, the factors which may elicit directed migration of amphibian leucocytes to specific areas of antigenic challenge are not known. The work presented in this chapter addresses the question of whether or not the directional motility of Rana temporaria leucocytes can be influenced by factors produced in response to antigenic or mitogenic challenge of immune cells in vitro.

Materials and Methods

Chemotaxis assays:

Two assay systems were used, to study the chemotactic responses of thioglycollate induced peritoneal exudate cells. The PEC were harvested, as described in the previous chapter, and washed three times prior to being used in the assays.

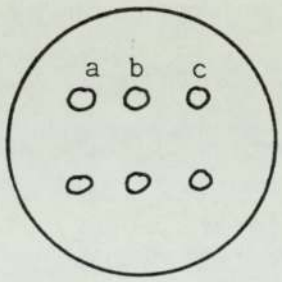
The Agarose gel assay:

The method described by Nelson Quie and Simons (Nelson, Quie and Simons 1975) was employed, in which cells migrate from a well cut into an agarose gel, towards wells containing chemotactic stimuli. The assay was performed in sterile 60 x 15mm petri dishes.

Agarose gel was prepared in 100ml volumes, by dissolving 0.8g of agarose(BDH) in 38ml of sterile DD H_2O in a boiling water bath for 20 minutes. The gel was transferred to a 48°C water bottle

and mixed with 63ml of Iscoves medium (Flow), supplemented with 1% FCS (Flow) Penicillin and Streptomycin (Flow) and L-Glutamine(Flow) which had been prewarmed to 48°C. The mixture was delivered to the petri dishes in 10ml aliquots using a prewarmed sterile pipette. The gels were then allowed to set on a level table at room temperature and then stored at 4°C until used.

For each assay, two, triplicate rows of wells 2.5mm in diameter and 2.5mm apart, were made in the gel, as shown below.



The wells were cut using a pasteur pipette, and the gel plugs removed using a hypodermic needle. Fluid which seeped into the wells, from the gel, was removed prior to the assay.

The centre well, b, received 10µl of a PEC suspension which contained 2×10^5 cells, and wells a, and c, received 10µl of the chemoattractant under test, and an appropriate control stimulus, respectively. The plates were covered, and incubated for 18 hours at 25°C and 95% humidity.

Following incubation, the plates were flooded with 5ml of methanol for 30 minutes, followed by 5ml of 10% formalin for 30 minutes. This procedure served to fix the cells, and also cleared the gel enabling observation of cells migrating between the gel and the plate surface. Plates were coded and read independently by two separate observers. The scores allocated were 'High', for a strong differential

migration towards the chemotactic stimulus; 'Moderate', for differential migration towards the stimulus; and 'None' for no differential migration. It was not possible to quantify the extent of cell migration accurately since irregularities in the cut gel, the dark field lighting conditions, and differential focusing required to visualise the cells, made precise measurements of the distances travelled by cells impossible.

To facilitate measurements, attempts were made to remove the gel and to stain the PEC adhering to the plate surface. However, cell adhesion on several batches of petri dishes used was very poor, and hence the subjective scoring system was routinely employed.

The Micropore Filter Assay:

In an attempt to more precisely quantify the chemotactic response, a modified Boyden chamber assay was used (Boyden, 1962) in which cells migrate from one compartment, through a micropore filter, towards a compartment containing chemoattractants. In the method employed here (after Neumann and Sorg, 1980) the two compartments were formed by the wells of two microtitration plates placed on top of each other, with cellulose acetate filters partitioning the wells (Fig.4.1).

Assay chambers were prepared by smearing petroleum jelly around the perimeter of both upper and lower test wells. The solutions to be tested were then introduced into the lower well, and the wells sealed with a 0.22μm filter. 5μm filtes were then placed above each 0.22μm filter and the upper plate was inverted and placed over the lower plate. Both plates were then clamped tightly together. 0.3 ml of PEC were then introduced into the upper walls, through an aperture made by using a hot needle to penetrate the plastic wall of the upper chamber. 5×10^5 cells were introduced into each upper chamber, this number giving an adequate cell density on the filter surface. The whole plate was then covered with aluminium foil, to

reduce evaporation of liquid, and then placed in a humid incubator at 25°C for 18 hours.

Following incubation, the fluid was removed from the upper wells using a syringe and needle and replaced with 0.3 ml of methanol, to begin fixation of the cells. After 20 minutes the methanol was removed and the two plates separated. Both filters were then fixed in formalin for 20 minutes and stained in Delafields Haematoxylin

The numbers of cells emerging at the lower surface of the 5 μm filter were determined as the mean count of cells in five randomly chosen high power fields of view under the microscope.

A cell was counted if, whilst focusing on the filter matrix, any part of that cell was clearly visible. This procedure includes any cells just emerging from the filter pores and those that have completely emerged. The number of cells adhering to the 0.22 μm filters were also determined, to assess the number of cells that had completely traversed the upper filter, and had become detached. Fewer than two cells per filter were normally seen, and were thus discounted.

Experimental Design

In both gel and filter assays, the chemotactic responses were determined by comparing the cell migration patterns induced in the presence of different stimuli outlined below.

a) Casein: Casein has been shown to be a potent chemoattractant for mammalian lymphoid cells (Wilkinson, 1972). A 10% solution of casein was therefore employed to test the responsiveness of amphibian cells in the two assay systems. Migration of PEC towards casein solutions was compared with migration towards culture medium alone.

b) Direct Stimulation of Spleen Cells with Antigen or Mitogen:

Several protocols were employed in order to stimulate spleen cells, cultured in the lower chamber of the assay apparatus, to produce chemotactic factors. 2×10^5 cells were introduced into each assay well prior to stimulation. The chemotactic activity of FSRBC-sensitized cells challenged with FSRBC in culture, was compared with that of FSRBC-sensitized cells alone, and also with FSRBC and tissue culture medium.

In addition, experiments were performed using non-immune spleen cells preincubated with PHA for four hours and then washed free of unbound PHA. Alternatively spleen cells were incubated directly in the lower culture chamber with PHA. PHA, at the dose employed, was also compared with culture medium for any chemotactic effect.

c) Spleen Culture P and R Supernatants

Both antigens and mitogens were used to generate P and R supernatants as described in the previous chapters. The P supernatants, containing products of cell activation, were compared with the control R supernatant for the presence of chemotactic activity. The use of supernatants enabled the partial physicochemical characterization of chemotactic activity. Sephadex gel filtration was used to separate con A induced P and R supernatants into four fractions (as previously described). These fractions were compared for chemotactic activity in the modified Boyden chamber assay. Sephadex separation also had the advantage of removing all the con A present from the supernatants.

Supernatants were added either undiluted for the gel assay in 10 µl volume, or diluted 1:2 in the modified Boyden Chamber filter assay, in 0.3 ml volume.

Each chemotactic stimulus and its appropriate control, were assayed at least twice in each experiment, unless otherwise indicated. In the filter assay the mean numbers of cells per high power field of the two replicates were compared for chemotactic stimulus and control using Student's 't' test (Appendix 2). The chemotactic effect obtained was expressed by a chemotactic index calculated as:

$$\text{Chemotactic Index} = \frac{\text{mean number of cells per h.p. field with chemotactic stimulus}}{\text{mean number of cells per h.p. field with control stimulus}}$$

Results

The Agarose Gel Assay:

Table 4.1 gives the results achieved using the agarose gel assay. The most effective chemotactic stimulus assayed was the 1% casein solution with 3 out of 4 experiments giving a high chemotaxis score, thus confirming its function as a potent chemoattractant for amphibian cells. A clear differential migration of cells towards FSRBC sensitized spleen cells incubated with FSRBC, when compared to FSRBC sensitized spleen cells alone, was also observed. FSRBC alone had no chemotactic effect, thus implying that the chemotactic response observed was due to interaction of the antigen and spleen cells. A soluble mediator was implicated in the attraction of the cells because the cells in the assay wells were not in physical contact. This was confirmed using cell-free P & R supernatants, when differential migration of cells was seen towards FSRBC P supernatants. HGG generated P supernatants were not as effective as chemoattractants, since only two out of four experiments showed a positive effect. PHA P supernatants were slightly more effective with positive results in three out of four experiments. Con A stimulation of spleen cells gave one high and one medium chemotaxis score.

Taken overall, both antigenic and mitogenic stimulation of spleen cells was capable of generating P supernatants which were chemotactic for PEC, when compared to the appropriate control R supernatants.

The Micropore Filter Assay:

The use of the filter method enabled the degree of chemotaxis to be more readily quantified and thus facilitated statistical analysis. Table 4.2 gives the results of experiments to determine the chemotactic effect of 1% casein solutions. Results are presented as the mean number of cells per high power field on the lower surface of the filter. For each experiment this value is derived from the mean cell count taken from two different filters; the value for each filter being obtained from 5 separate measurements. The chemotactic index, which was derived from the number of cells moving towards the casein and those moving towards medium only, is also given. Experiments employing casein gave a significant chemotactic effect, with chemotactic indices of 2.4 and 5.2 respectively, thereby confirming the chemotactic nature of casein observed in the gel assay. The appearance of stained filters for both casein and control filters is illustrated in Fig. 4.2 Both polymorphonuclear and monocytic cell types are visible, although PMN dominated most of the filters studied. This may reflect a faster migration rate for PMN.

Table 4.3 shows the results of experiments in which spleen cells were stimulated directly in the lower chamber of assay plates. In vitro stimulation of FSRBC-sensitized spleen cells with FSRBC generated significant chemotactic activity when compared to spleen cells alone, with chemotactic indices of 2.6, 1.5 and 2.1 respectively.

FSRBC alone were not chemotactic, showing a chemotactic index of 0.9 when compared to culture medium only.

An equivalent experiment in which spleen cells were stimulated with PHA gave less clear cut results. When PHA was included in the lower well with the spleen cells, a chemotactic index of 0.6 was obtained, PHA alone giving a lower index of 0.4. When spleen cells were pre-incubated with PHA for 4 hours, washed and then introduced to the lower well, a chemotactic index of 4.2 was seen. The most likely explanation for the observed reduction in cell movement when PHA was present in the wells, is probably that the PHA agglutinates the test cells in the upper chamber preventing their free migration through the filter. PHA stimulated spleen cells which were washed to remove free PHA from the culture were able to produce chemotactic activity following mitogenic triggering.

Only P and R supernatants derived from con A stimulated spleen cells were studied for chemotactic activity in the filter assay. Whole supernatants gave a chemotacti c index of 2.0. These supernatants were also separated on Sephadex into four fractions and these fractions compared for chemotactic activity. Fig. 4.3 gi ves the results for each separated supernatant. The mean chemotactic indices for each fraction are given in Fig. 4.4.

Although the absolute numbers of migrating cells are different for the two different supernatants, the overall pattern of the two graphs comparing P to R fractions is remarkably constant. Only fraction II, containing material in the range 16-27,000 Dalton gave significant differential cell migration, with chemotactic indices of 4.3 and 2.3 respectively. Fractions I, II and IV gave C.I. which were not significantly higher or lower than unity. The overall effect of fraction II, mean C.I. 3.3 was actually higher than that of whole supernatant which may imply that some inhibiting

activity is present in other fractions of the whole supernatant.

Discussion

The results presented in this Chapter demonstrate that Rana temporaria peritoneal cells can respond by directional migration towards antigen or mitogen stimulated spleen cells, or towards the supernatants derived from cultures of stimulated cells. The demonstration of a chemotactic effect following stimulation of lymphoid cells, as with migration inhibition factor in Chapter 3, does not of necessity prove that a lymphokine is involved. In fact, a number of physiological mechanisms exist in mammals that can lead to the generation of chemotactic factors. These include Antigen/antibody complexes, clotting system components, complement system components, leukotrienes and bacterial products. These mechanisms should be excluded before a lymphocyte derived chemotactic factor (LDCF) is invoked.

Sephadex separation of the con A supernatant indicates that Rana Chemotactic factor has a molecular weight bwtween 16 - 27,000 Daltons. This size range serves to exclude most of the above factors. Thus ag/ab complexes are of a higher MW (> 100,000) and bacterial chemotaxins leukotrienes and the clotting system components fall into a lower MW range (reviewed in Snyderman and Mergenhagen 1976). Only C5a of the complement components has an appropriate MW (17,000 Daltons in mammals). A role for this may however be excluded as the foetal calf serum used was routinely heat inactivated, and hence any complement would have to be endogenously synthesized, by splenic macrophages and then activated to C5a only in stimulated cultures. Antibody mediated complement activation is unlikely since no agglutinating antibody could be detected in any of the supernatants studied.

If the factors considered above are excluded, then consideration must be given to the possibility that the chemotactic effects achieved are as a result of the production of a chemotactic lymphokine.

The results of Sephadex fractionation are much clearer than for fractionated MIF in the preceding chapter, in that chemotactic activity is only present in fraction III, this corresponds to a MW within the range of 16-27,000 Daltons. Other species studied have given a wide range of MW values for LDCF, e.g. Human LDCF 12,500 and 25,000; Guinea Pig 12,500 and 60-20,000; Mouse 40,000, Rat 30-60,000 and Chicken 12,500 Daltons (reviewed in Altman, 1978). Rana CF therefore has a direct counterpart in the Human 25,000 Dalton lymphokine. It has been suggested that lymphokines may be derived from a common molecule of MW 12,500 - 15,000 (Yoshida, 1979) this common molecule has been termed "mother factor". For CF this mother factor would have a MW of 12,500 the association of similar factors would give rise to MW which were based on multiples of 12,500. If this theory is correct, then Rana LDCF may exist as a dimer.

The mechanism of action of chemotactic factors on target cells has not been clarified in mammals, but it has been linked to the hydrophobicity of the molecules involved. Thus the addition of hydrophobic groupings to the chemotactically inert bovine serum albumin leads to the formation of a progressive more chemotactic molecule (Wilkinson and McKay, 1972). Casein is a protein which has a high number of hydrophobic groupings. These are mainly hidden in the interior of the molecule by folding of the structure, however, not all can be accommodated inside, and some are left exposed. In mammalian cells this surface hydrophobicity is thought to be the source of casein's chemotactic properties. That casein maintains its activity for Rana cells implies that similar mechanisms may be operational at the amphibian level of evolution.

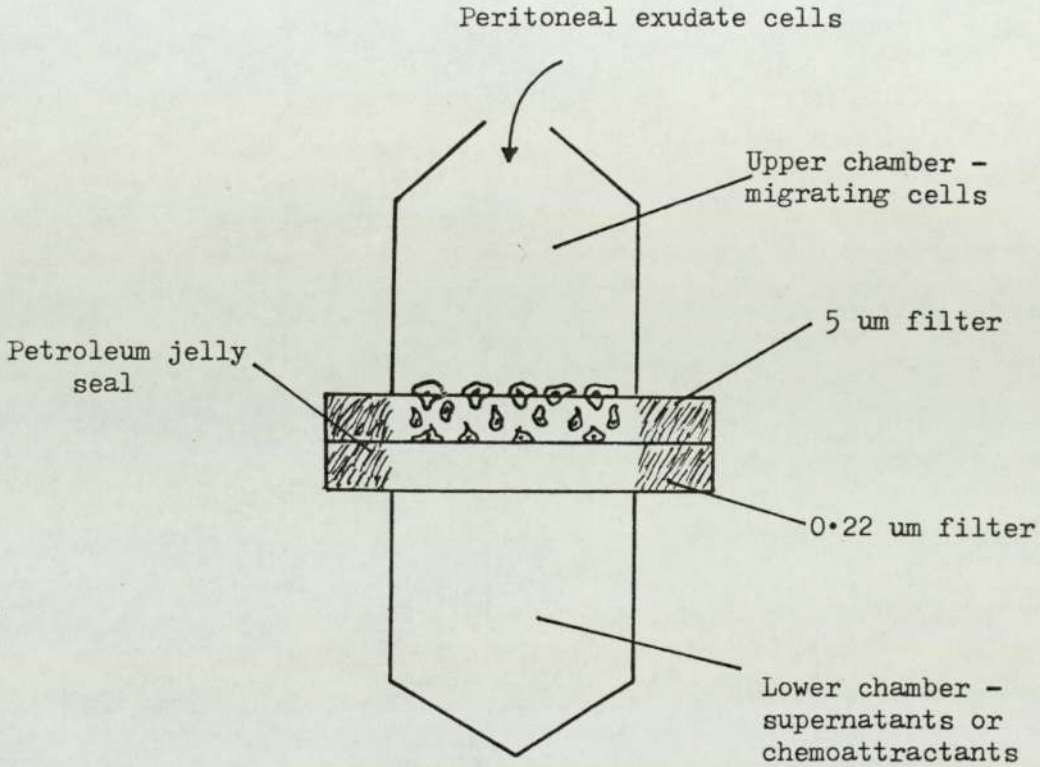
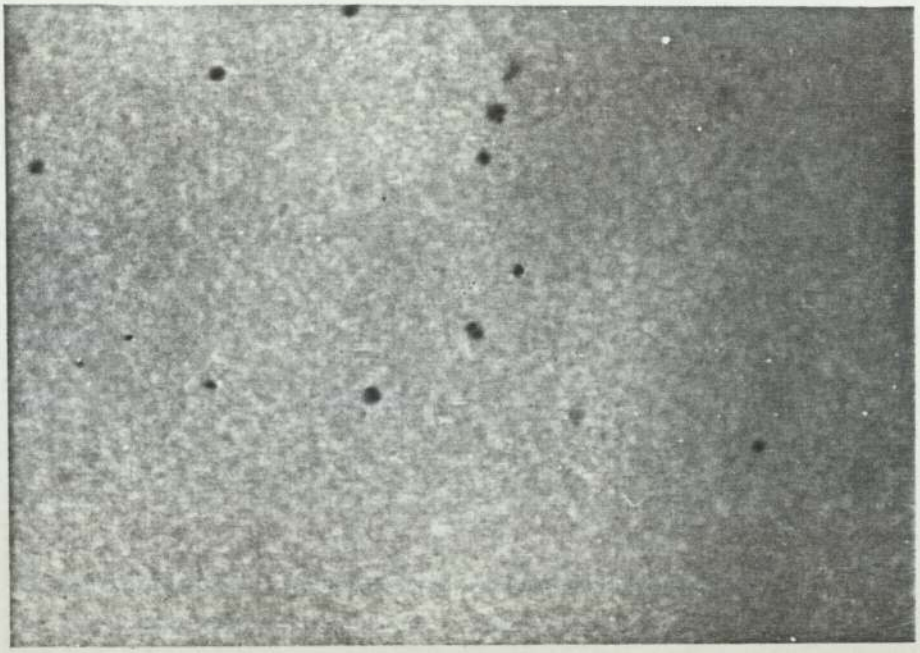


Fig.4.1 Diagram of the modified Boyden chamber filter assay, for the measurement of chemotaxis.

a)



Scale 100 μm

b)

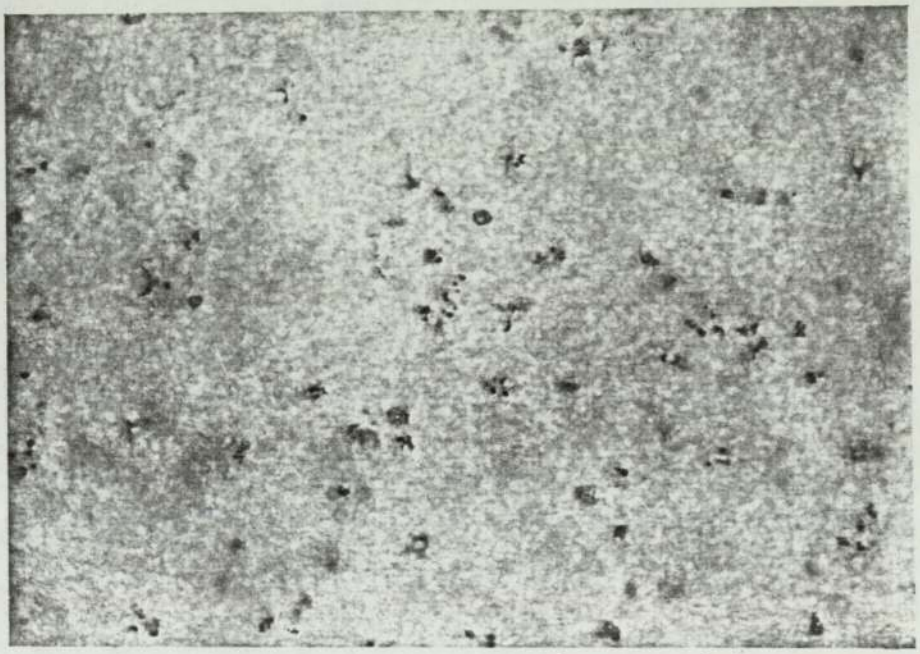


Fig. 4.2 The appearance of stained chemotaxis filters from control (a) and casein supplemented (b) Boyden chamber wells. The photographs illustrate a high power view of the lower surface of the filters, through which PEC have been allowed to migrate, for 18 hours, towards the lower chamber. In (a), the lowerwell contained tissue culture medium only, and in (b) it contained a 1 % solution of casein in identical medium. Staining was in Delafields haematoxylin.

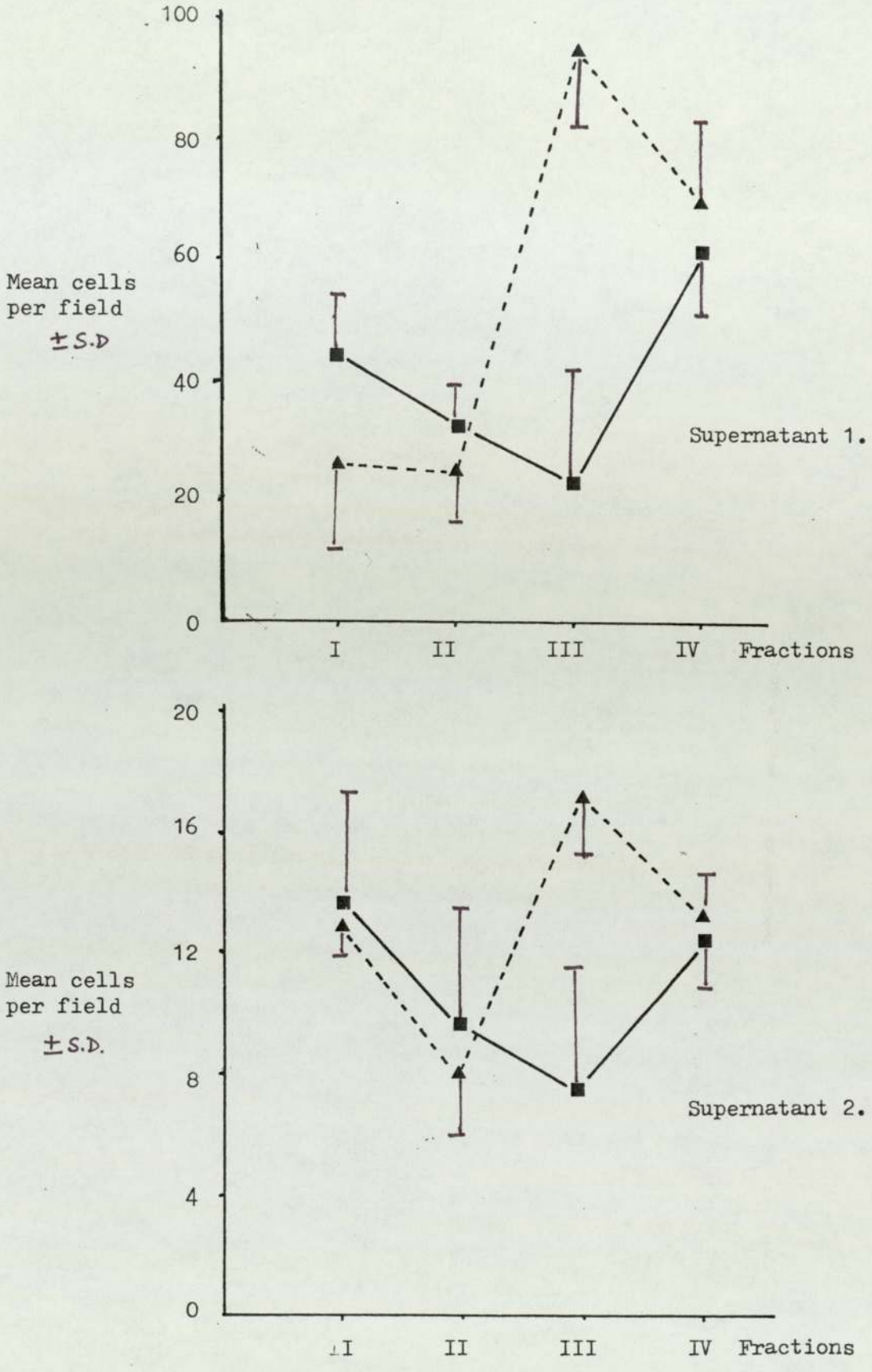


Fig. 4.3 Comparison of sephadex fractions of con A-induced P and R supernatants for their effects on the directional migration of PEC. The results are expressed as the mean of 5 high power microscope fields, from 2 separate filters, for each point. Only cells in focus at the lower surface of the 5um filter , after 18 hours in culture, were included in the count.

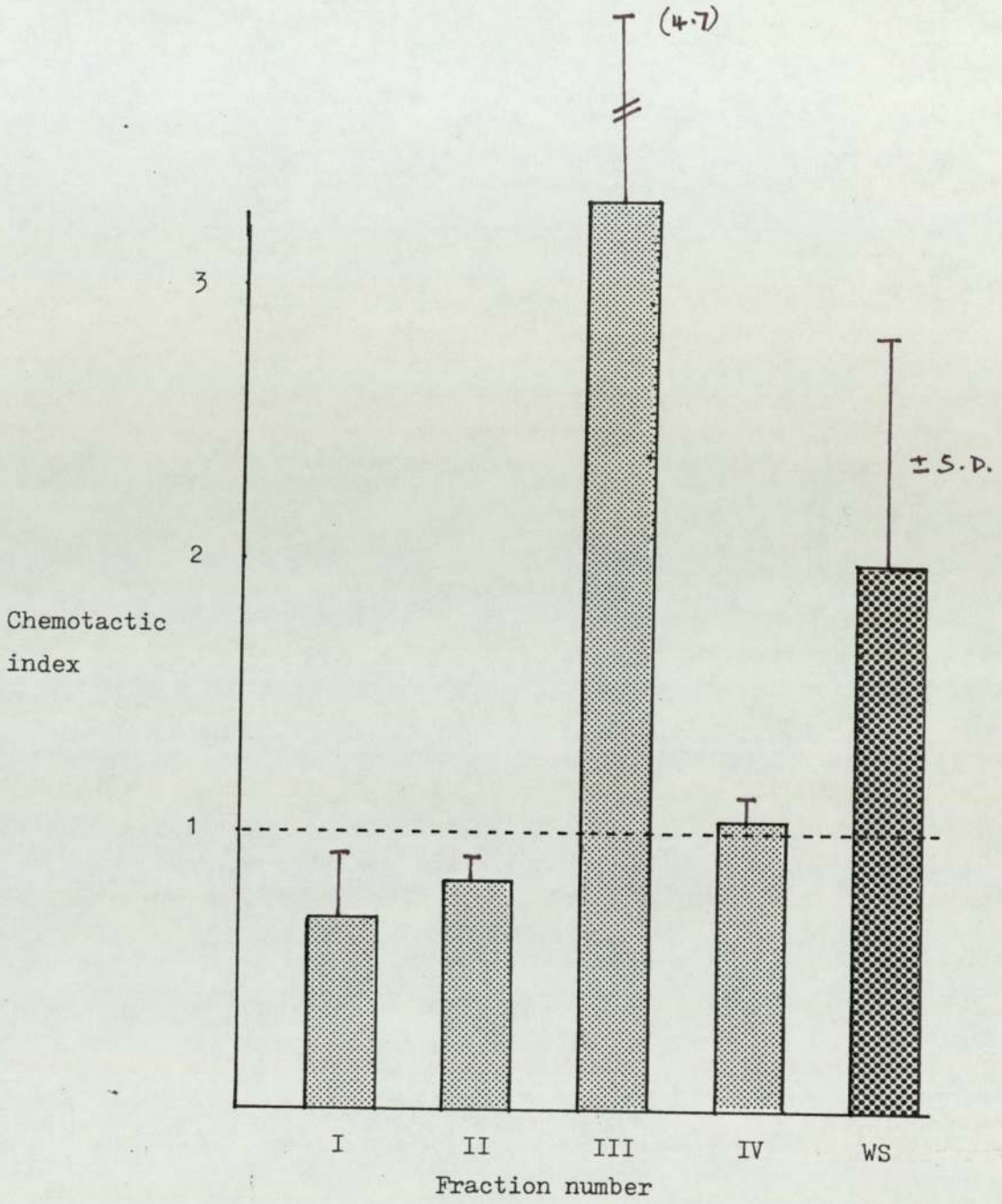


Fig. 4.4 Summary of the chemotactic effects of sephadex fractions of con A-induced supernatants. The chemotactic indices are derived from the results given in Fig. 4.3, and compare the mean numbers of cells migrating towards P supernatant fractions, with those migrating towards the corresponding R fractions. A chemotactic index greater than one, indicates differential cell movement towards the P supernatant fractions. The WS column compares con A supernatants prior to fractionation.

TABLE 4.1 The chemotactic behaviour of peritoneal exudate cells in the agarose gel assay

Chemotaxis scores allocated were as follows:

High: :- Strong differential movement towards the chemotactic stimulus

Medium :- Differential movement towards the chemotactic stimulus

None :- No differential migration

Each symbol I, represents the score for an individual experiment

Chemotactic stimulus	Control stimulus	Chemotaxis scores None	Medium	High
1% Casein	Medium only	0	I	III
FSRBC sens. spleen cells + FSRBC	FSRBC sens. spleen cells only	I	IIIII	II
FSRBC P super	FSRBC R super	0	II	0
HGG P super	HGG R super	I	I	0
HGG P super	HGG R super	I	I	0
PHA P super	PHA R super	0	II	0
PHA P super	PHA R super	I	I	0
con A P super	con A R super	0	I	I

TABLE 4.2 The chemotactic effect of casein on the migration of peritoneal exudate cells in the Boyden chamber filter assay. The mean number of cells per high power microscope field was derived from five separate counts from each of two separate filters.
The chemotactic index compares the mean number of cells migrating towards casein, with those migrating towards medium alone. Student's 't' test was used to determine the significance of the difference.

	Mean No. Cells per H.P. field	Chemotactic index	Significance of difference
1% Casein	41 ± 12	2.4	0.05
medium only	17 ± 4		
1% Casein	100 ± 16	5.2	0.01
medium only	19 ± 10		

TABLE 4.3 The chemotactic movement of PEC in response to stimulated spleen cells in the Boyden chamber filter assay.

	Mean No. cells per H.P. field	Chemotactic index	Significance of difference
Spleen cells + PHA	11 ± 5	0.6	NS
Spleen cells only	18 ± 4		
PHA pulsed spleen cells	47 ± 19	4.2	0.01
Spleen cells only	11 ± 4		
FSRBC sens.spleen cells + FSRBC	30 ± 14	2.6	0.05
FSRBC sens. spleen cells only	12 ± 3		
FSRBC sens. spleen cells + FSRBC	53 ± 11	1.5	0.05
FSRBC sens. spleen cells only	35 ± 9		
FSRBC sens. spleen cells + FSRBC	37 ± 8	2.1	0.01
FSRBC sens. spleen cells only	17 ± 3		
FSRBC	18 ± 3	0.9	NS
PHA	9 ± 5	0.4	NS
Medium only	22 ± 5		

Results expressed as mean +/- one standard deviation, the significance of the difference being determined by Student's 't' test. The chemotactic index compares mean Nos. of cells in the stimulated cultures with their appropriate control.

CHAPTER FIVE

STUDIES OF ANTIGEN AND MITOGEN INDUCED FACTORS AFFECTING THE PROLIFERATION OF SPLEEN CELLS

Introduction

The factors investigated in the preceding chapters were involved in altering the behavioural properties of inflammatory cell types. By far the greater number of mammalian lymphokines, however, act to influence the proliferation or differentiation of other lymphocytes (Waksman and Namba, 1976) (Waksman, 1979). Initial reports in 1965 showed that supernatants from alloantigen activated lymphocytes contained material which induced fresh lymphocytes to divide (Kasakura and Lowenstein, 1965) (Gordon and Maclean, 1965). Maini also described a soluble mitogenic factor in supernatants of antigen stimulated leucocytes (Maini, Bryceson, Wolstencroft and Dumonde,1969). As with other lymphokines, this lymphocyte mitogenic factor (LMF) can be produced by antigen or T cell mitogen stimulation, but affects the proliferation of other lymphocytes, regardless of antigen specificity or MHC type (Levis, Whalen and Miller,1974) (Wolstencroft, Maini and Dumonde,1976).

Evidence for the production of such mitogenic lymphokines is limited in non-mammalian species. Chicken leucocytes, when cultured with antigen, have been shown to release a soluble LMF (Oates, Bissenden, Maini, Payne and Dumonde, 1972). Both thymectomized and bursectomized chickens were shown to produce a soluble mitogenic activity, but the authors suggest that only the T cell product was a true lymphokine, the B cell product consisting of antigen/antibody complexes, which are also known to stimulate lymphocyte proliferation. (Moller 1969). In mammals it has been shown that LMF is exclusively a T cell product, whereas other lymphokines, such as MIF, may be

produced by B cells as well (Rocklin, MacDermott, Chess, Schlossman and David, 1974). The only report of a mitogenic factor occurring in invertebrate species comes from LeClerk and co-workers who have demonstrated a soluble factor, in the supernatants of Poke Weed Mitogen stimulated axial organ cells of starfish, which increases the proliferation of other axial organ cells in vitro (Brillouet, Luquet and LeClerc, 1981). This activity could only be detected in concentrated supernatants.

In Amphibia, the evidence for LMF is circumstantial, or negative. Supernatants from mixed lymphocyte cultures of Xenopus laevis, undergoing a strong proliferative response, have been examined for mitogenic effects, but these were not found. (Johari, Botham and Manning, 1981). Such strong proliferations are routinely observed in amphibian leucocyte cultures responding to antigens and allo-antigens. This proliferation has been linked in mammals to the production, by antigen reactive cells, of LMF which then stimulates many other lymphocytes to divide (Wolstencroft, Maini and Dumonde, 1976).

To investigate this issue in amphibia, the supernatants from both antigen and mitogen stimulated Rana spleen cell cultures were compared with control supernatants for their effect on the proliferation of fresh spleen cells. Sephadex fractionation of con A induced supernatants was also carried out in an attempt to partially characterize and regulatory activities present.

Materials and Methods

Assay for the proliferation of Splenocytes:

The uptake of tritiated thymidine by a population of cells has been shown to correlate with the extent of their proliferation as determined by other methods (Gunther, Wang and Edelman, 1974).

This assay system is routinely used by many workers to assess the proliferation of amphibian lymphocytes (Wright and Cooper, 1979) (Weiss and Du Pasquier, 1973) and has been employed in the experiments described in this thesis.

The assays were performed in sterile-well microtitration plates, each well receiving 2×10^5 washed spleen cells in a final volume of 100 μl culture medium containing 1% foetal calf serum. Such conditions have been shown to result in good stimulation of amphibian lymphocytes. (Weiss and Du Pasquier, 1973). The cells were incubated for 48 hours at 25°C and 95% humidity. Each well was then pulsed with 1 μCi of tritiated thymidine (specific activity 5 Ci/mMol, Amersham) in 10 μl volume, and returned to incubate for a further 18 hours. Following incubation, the cells were flushed from the wells using a Skatron semi-automatic cell harvester (Flow Laboratories) and deposited on to individual glass fibre filter discs, any radioactive thymidine in solution being washed away. The filters were dried at 60°C for 1 hour, and then transferred to individual glass vials. The cellular material was digested for 1 hour at 60°C in 0.5. ml of hyamine hydroxide (B.D.H.) 5 ml of scintillation cocktail (PPO and POPOP in Toluene) was added to each vial. Vials were then allowed to stand for several hours before counting. Incorporated tritiated thymidine was estimated by scintillation counter, calibrated to give a reading corrected to disintegrations per minute of β emitting activity in the vial, and hence in the harvested cells. This method avoids the use of methanol and tricholoracetic acid, and has been shown to achieve effective harvesting of tritiated thymidine labelled mammalian lymphocyte cultures (Hirschberg and Thorsby, 1973).

The extent of increase in proliferation of spleen cells following stimulation, is given as a stimulation index (S.I.) which compares the mean D.P.M. in stimulated culture

wells with the mean D.P.M. in non-stimulated, control cultures. In each case, the mean D.P.M. is determined from triplicate wells and S.I. are only calculated for spleen cells from individual animals, thus avoiding any allogeneic stimulation in these outbred animals.

Experimental Design

Both antigen and mitogen stimulation were employed, in initial experiments, in order to assess the efficacy of the technique. S.I. were calculated from the following formula:

$$SI = \frac{\text{Mean DPM in ag or mitogen stimulated speen cell cultures}}{\text{Mean DPM in non-stimulated speen cell cultures}}$$

Spleen cells from animals immunized 14 days previously with HGG gave a SI of 3.1, when comparing tritiated thymidine incorporation by cells challenged in vitro with 100μ g ml^{-1} HGG and cells from the same animals in culture medium alone. HGG had no effect on the proliferation of non-sensitized cells (SI 1.1). Similar stimulations were observed when the T cell mitogens PHA and con A were used. At its optimal concentration of 50μ g ml^{-1}, PHA gave a SI of 5.6 and CON A at 1 μ g ml^{-1} a SI of 3.4. The B cell mitogen LPS gave a SI of only 1.5, even at the highest concentration used, 2000 μ g ml^{-1}. These results thus confirmed that the assay system used could detect a mitogenic response in spleen cells under the culture conditions used.

Spleen culture P and R supernatants, generated, by antigen and mitogen as described in previous chapters, were used to supplement the assay wells, in order to investigate the existence of any soluble mitogenic activity. Each of triplicate

wells received 50 µl of spleen cell suspension, containing 2 x 10^5 cells, and 50 µl of supernatant. A SI comparing DPM in P with DPM in R supernatant supplemented cultures was determined. The coefficient of variation (Standard deviation ÷ mean) for DPM in control cultures ranged from 0.03 to 0.5 (mean value 0.3). Stimulation was, therefore, considered to be significant if a SI of greater than 1.6 was obtained.

Nine P and R supernatants, indicated below, were tested for their effects on the proliferation of spleen cells from individual animals. Each supernatant was tested on spleen cells from at least two different animals. All supernatants were assayed at 1:2 final dilution, except C which was tested at 1:4.

Supernatants:

A, B and C	-	HGG P+R from 14 day sensitized cells
D	-	SRBC P+R from 14 day sensitized cells
E	-	FSRBC P+R from 14 day sensitized cells
F	-	PHA P+R from non sensitized cells
G, H and J	-	CON A P+R from non-sensitized cells.

Supernatant J was fractionated on Sephadex G100-120 as described previously, to give four fractions (I and IV), with MW ranges indicated in Fig.5.1 Each fraction from both P + R was assessed for its effects on proliferation. Proliferation in R supplemented wells was found not to differ from that in non-supplemented wells

Results

a) The effect of antigen induced supernatants on spleen cell proliferation

Results expressed as mean DPM are given in Tables 5.1 and 5.2. Using supernatants from HGG-sensitized spleen cells, the extent of cellular proliferation was greater in cultures supplemented with P rather than R supernatants, only one culture gave an S.I. of less than one. The mean SI for supernatant batches A and B were 1.6 and 1.9 respectively, indicating the presence of some mitogenic activity in the P supernatants. The lower SI of 1.4 observed using supernatant C, may reflect the greater dilution employed rather than the lack of any mitogenic activity.

Supernatants from RBC immunized spleen cultures gave differing results. The SRBC-generated supernatant (D) gave no differential effect for P and R (mean SI 1.1). The FSRBC P supernatant (E) however, caused markedly increased proliferation of the test spleen cells, when compared to R, with a mean SI of 2.9. The problems inherent in using wild animals, whose state of immunological reactivity may vary, are shown by the variable responses of the four different animals, to an identical supernatant in identical culture conditions. Thus stimulation indices range from 7.4 down to 0.4, with mean DPM of from 94 to 1,649 for R supplemented wells. The combined results clearly indicate that the in vitro challenge of in vivo sensitized spleen cells, by the sensitizing antigen, can lead to the formation of soluble material which is mitogenic for other non-sensitized spleen cells.

b) The effect of mitogen induced supernatants on spleen cell proliferation.

Both PHA and conA were used to generate supernatants used in these experiments, the results for whole supernatants are given in Table 5.3. PHA-generated P supernatants gave clear

mitogenic effects when compared to R. SI ranged from 1.9 to 2.6 with a mean value of 2.3 Con A supernatants were found to give the reverse effect, in fact proliferation was reduced in cultures when P supernatant was added. The results of 7 experiments with two different supernatants gave only one case when a SI greater than 1 was seen. The mean SI for each of the two batches was 0.6. This suppressive effect is surprising, considering that the P supernatant is derived from spleen cultures, which are undergoing a proliferative response to the con A (SI 3.4). In order to further investigate this phenomenon, a batch of con A supernatant (J) was subjected to Sephadex fractionation. The results of applying the resulting P and R fractions to spleen cell cultures are given in Table 5.4 and summarized in Fig.5.1 Suppressive activity was found to be present in both the high and low MW range (fractions I and IV) with mean SI of 0.6 and 0.3 respectively. In the mid range (fractions II and III) however, mitogenic activity could be detected, with mean SI of 4.0 and 4.1.

Thus, although PHA stimulation of spleen cells gives rise to the production of soluble mitogenic activity, con A P supernatants induce suppression of proliferation. Con A is used in mammalian work to generate T suppressor cells (Waltenbaugh 1979) and so, con A stimulation may be inducing suppressor activity in the spleen cultures employed here. The fact that both stimulatory and suppressive components are present in the con A P supernatant, indicates that con A may be having a dual effect: that it has a direct mitogenic effect is shown by the proliferation seen in the original cultures used to make the supernatant. In addition, it may induce a suppressive component which would eventually prevent proliferation. The mitogenic component of the supernatant

may be identical to that produced by PHA stimulation, its effects being masked by the suppressive material. Such dual opposed activities have been detected in mammalian lymphokine preparations. (Harwell, Kappler and Marrack 1976).

Discussion

Results presented in this chapter demonstrate that Rana temporaria spleen cells, which are undergoing a proliferative response to T cell dependent, soluble or cellular antigens, or to the T cell mitogen PHA, can elaborate soluble material which is mitogenic for other spleen cells. Similar findings, when originally reported by Maini and others, led to the postulation of a lymphokine, lymphocyte mitogenic factor, produced in their case by antigen stimulation of human lymphocytes (Maini, Bryceson, Wolstencroft and Dumonde,1969). Subsequent authors have demonstrated a similar lymphokine in a variety of species (reviewed in Wolstencroft Maini and Dumonde,1976) which it seems may now include Rana temporaria.

Con A has been shown to be a potent inducer of LMF from mammalian leucocytes. (Mackler, Wolstencroft and Dumonde 1972). However, production of LMF was shown to be maximal at 24 hours, - activity being lower at later times. In addition, con A is routinely used as an inducer of T suppressor cells, where optimally mitogenic concentrations induce maximal numbers of suppressor cells (reviewed in Hume and Weidemann 1980). Supernatants from such con A induced Ts cells have been found to contain two different soluble suppressive factors: soluble immune response suppressor or SIRS (Rich and Pierce 1974) and inhibitor of DNA synthesis or IDS (Folch and Waksman 1974) The conditions employed to stimulate

Rana temporaria spleen cells in this study, (72 hour culture with optimum dose of con A) may have been expected, by analogy with mammalian results, to yield suppressor activity. The observed suppressive supernatants would seem to confirm that similar processes may be operating in the frog, and if so, that T suppressor cells are indeed present at the amphibian level of evolution.

The Sephadex fractionation of the con A supernatant revealed suppressive activity of MW above 50,000 Daltons, and also of from 9-16,000 Daltons. There is no mammalian equivalent for the lower MW material, however, both SIRS, MW 48-67,000 and IDMS MW 80,000 Daltons (Hume and Weidemann, 1980) would fit the high MW range.

SIRS is thought to work by inducing macrophages to release a further soluble suppressor factor which non-specifically suppresses immune responses in vitro, (Aune and Pierce, 1981). Interestingly, SIRS cannot be physicochemically separated from MIF. the effect of SIRS may then be due to the activating function of MIF on macrophages causing the release of factors which are suppressive for lymphocyte proliferation. Such a mechanism, whereby the stimulation of lymphocytes leads to the eventual production by macrophages of a factor, which suppresses lymphocyte responses, could provide a useful feedback control of immune responses. The sephadex fraction I which contains the Rana suppressive material, also contains high MIF activity, which may imply that the suppressor is acting on macrophages in an analogous fashion. SIRS normally acts to suppress antibody responses. However, IDS directly influences the proliferation of T and B lymphocytes (Waksman, 1979) and thus an equivalent of IDS is more likely to be operating in the present study.

Mitogenic activity, induced by con A, was found in the MW range 16-50,000. Reports for mammalian LMF have given MW varying from 15,000 to 30,000 for humans, and from 20,000 to over 100,000 for guinea pigs, with most estimates being from 20 to 30,000 Daltons (reviewed in Farrar and Koopman, 1979). Thus, a LMF of MW 30,000 may be expected to show activity in fractions II and III (range 27-50,000 and 16-27,000 Daltons respectively). A choice of different MW range fractions in the present study may have revealed whether or not such a factor was present in Rana.

The presence of both mitogenic and suppressive lymphokines in the same supernatant is not unknown. Thus, some supernatants become stimulatory only on dilution, implying a suppressive material whose effects become diluted out (Harwell, Kappler and Marrack, 1976). In this study, the conditions used to generate the supernatant may have been such that although initial con A stimulation caused the release of LMF, enough time was allowed for the induction of suppressor cells, whose products masked the mitogenic effect. It remains to be determined therefore, whether or not the LMF present in the con A supernatants is the same as that seen in the PHA-and antigen-induced supernatants.

The demonstration of an amphibian LMF has great significance in the understanding of the evolution of immunoregulation. Thus, following antigen challenge, sensitized cells can release material which causes other non-sensitized cells to divide. This serves to amplify the original recognition-event and generates greater numbers of lymphocytes at the site of challenge. Additionally, it has been shown that contact with LMF can cause lymphocytes to release other lymphokines (Gately, Gately and Mayer, 1976) which once again is an amplificatory step. The invivo significance

of such amplification is obvious. Antigen recognition by a single lymphocyte would result in the release of LMF which in turn, would induce many other lymphocytes to release inflammatory mediators such as MIF, CF and further LMF. This would lead to the rapid accumulation of phagocytes and proliferating leucocytes, such as that seen in delayed type hypersensitivity reactions or allograft rejection.

Although evidence for such an in vivo role is limited, the presence of MF can be demonstrated in the draining lymph from the lymph nodes of immunized sheep undergoing antigen challenge, and also in the synovial fluid of arthritic joints (Neta and Salvin, 1981). An in vivo function for Rana MF remains to be investigated

The preceding chapters have established that amphibian leucocytes can produce factors which are equivalent to mammalian inflammatory lymphokines. This chapter demonstrates that regulatory factors can also be produced, although the phylogenetic and functional relationships between these factors and the many different mammalian lymphokines is unclear.

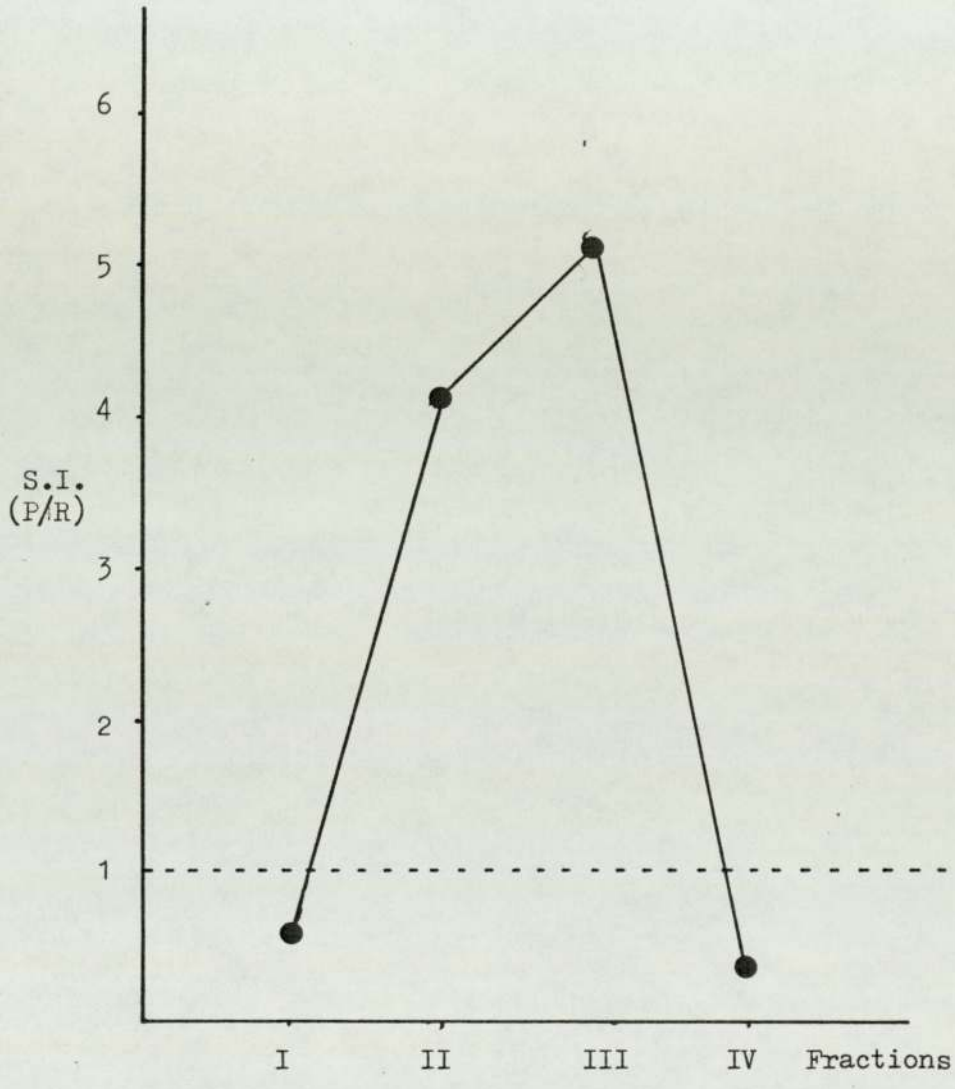


Fig. 5.1 Comparison of sephadex fractions of con A-induced, P and R supernatants for their effects on the proliferation of spleen cells. The stimulation indices are derived from the results given in Table 5.4, and compare the mean DPM recorded for spleen cells cultured in P supernatant fractions, with DPM in R supernatant fractions. A S.I. of greater than one, indicates higher tritiated thymidine uptake in P supernatant cultures.

MW - ranges were > 50,000 for fraction I, 27 - 50,000 for fraction II, 16 - 27,000 for fraction III and 9 - 16,000 for fraction IV.

TABLE 5.1 Comparison of HGG induced P and R supernatants for their effects on spleen cell proliferation.

Supernatant	Test animal	DPM P		DPM R		Stimulation index	
HGG A	1	2908	(401)	1675	(56)	1.7	
	2	656	(42)	341	(50)	1.9	$\bar{x}$ 1.6
	3	733	(255)	488	(99)	1.5	
	4	550	(119)	434	(52)	1.3	
HGG B	1	361	(68)	519	(67)	0.7	
	2	661	(95)	331	(67)	2.0	$\bar{x}$1.9
	3	679	(104)	303	(57)	2.3	
	4	510	(58)	192	(14)	2.7	
HGG C	1	854	(62)	529	(94)	1.6	$\bar{x}$1.4
	2	2440	(106)	2143	(240)	1.1	

Stimulation indices calculated as DPM P ÷ DPM R

Figures in parentheses are standard errors of the mean of triplicate counts.

$\bar{x}$ indicates the mean stimulation index for each individual batch of supernatants, A B or C.

TABLE 5.2 Comparison of RBC induced P and R supernatants for their effects on spleen cell proliferation.

Supernatant	Test Animal	DPM P	DPM R	Stimulation index	
SRBC D	1	1740 (164)	1597 (174)	1.1	$\bar{x}$ 1.1
	2	2017 (279)	1964 (156)	1.0	
FSRBC E	1	168 (21)	94 (11)	1.8	
	2	2733 (409)	370 (44)	7.4	$\bar{x}$ 2.9
	3	3322 (1490)	1649 (63)	2.0	
	4	223 (54)	619 (73)	0.4	

Stimulation indices calculated as DPM P ÷ DPM R

Figures in parentheses are standard errors of the mean of triplicate counts.

$\bar{x}$ indicates the mean stimulation index for each individual batch of supernatants, D and E.

TABLE 5.3 Comparison of mitogen induced P and R supernatants for their effects on spleen cell proliferation.

Supernatant	Test animal	DPM P	DPM R	Stimulation index	
PHA F	1	3290 (236)	1264 (410)	2·6	
	2	1388 (82)	742 (91)	1·9	$\bar{x}$ 2·3
	3	4920 (195)	1934 (267)	2·5	
CON A G	1	638 (104)	1342 (290)	0·5	
	2	252 (20)	538 (18)	0·4	$\bar{x}$ 0·6
	3	368 (112)	220 (51)	1·7	
CON A H	1	679 (21)	1373 (343)	0·5	
	2	281 (16)	423 (11)	0·7	$\bar{x}$ 0·6
	3	393 (83)	1002 (126)	0·4	
	4	348 (24)	376 (35)	0·9	

Stimulation indices calculated as DPM P ÷ DPM R

Figures in parentheses are standard errors of the mean of triplicate counts.

$\bar{x}$ indicates the mean stimulation index for each individual batch of supernatants, F,G and H.

Supernatants G and H were preabsorbed using sephadex beads prior to filtration, in order to remove any residual Con A.

TABLE 5.4 Comparison of sephadex fractions of con A induced P and R supernatants for their effects on spleen cell proliferation

Sephadex fraction	Test animal	DPM P	DPM R	Stimulation index	
J I	1	442 (99)	943 (32)	0·5	$\bar{x}$ 0·6
	2	301 (103)	488 (19)	0·6	
J II	1	679 (202)	489 (87)	1·4	$\bar{x}$ 4·0
	2	1602 (45)	242 (10)	6·6	
J III	1	269 (96)	146 (103)	1·8	$\bar{x}$ 5·1
	2	1688 (583)	203 (40)	8.3	
J IV	1	135 (47)	346 (101)	0·4	$\bar{x}$ 0·3
	2	197 (6)	763 (181)	0·3	

Stimulation indices calculated as DPM in P supernatant fractions ÷ DPM in R supernatant fractions.

Figures in parentheses are standard errors of the mean of triplicate counts.

$\bar{x}$ indicates the mean stimulation index for each individual fraction of supernatant J.

CHAPTER SIX

GENERAL DISCUSSION

In his prologue to a symposium on the phylogeny of immunogical memory, Hildemann reminds us of the "information gaps" that exist in the phylogenetic study of immunity, and also of the fact that many aspects of the mammalian immune system remain poorly understood (Hildemann, 1980). This is particularly true for the study of lymphokines and their involvement in immune reactions. Although a wide range of mammalian lymphokines has been described on the basis of diverse biological actions, the biochemical properties, relations between particular mediators, and their precise role, if any, *in vivo* remains unclear. In lower vertebrates, reports of lymphokine activities have been very sparse, and, in the main, restricted to demonstrations of direct migration inhibition phenomena. In the light of these considerations the present study would appear to be timely.

This thesis describes the first experimental evidence for the existence of a range of mediators, corresponding to mammalian lymphokines, in an ectothermic vertebrate. The assay systems employed and the properties of the mediators implicated in each system are summarized in Fig. 6.1. It can be seen that these *Rana* mediators fulfil the criteria used by Dumonde to define lymphokine activities (Dumonde, Wolstencroft, Panay, Matthew, Morley and Howson, 1969). Firstly, the factors described (with the possible exception of MStF in Chapter three) have been shown to be soluble, and do not appear to be either antibody or antigen antibody complexes. Secondly, by definition, all affect the behaviour of some cell type in the particular assay system

employed, and all are released following either specific antigenic or mitogenic stimulation, *in vitro*. This latter requirement for immune specificity implicates lymphocytes in their production. It would seem reasonable, therefore, to refer to the *Rana temporaria* mediators by using the names and conventions which have been applied to their proposed mammalian counterparts.

On the strength of the properties so far investigated, *Rana* lymphokines seem to strongly resemble mammalian lymphokines; they occupy similar molecular weight ranges, and are produced with similar kinetics under similar circumstances of stimulation. Evidence for phylogenetic conservation of structure and function is provided by the fact that ranid and mammalian MIF both display similar sugar inhibition profiles (to α- L - fucose), and that the *Rana* MIF retains its migration inhibition properties for rat PEC.

Such cross-class activity has also been shown by Sipka, Boldogh and Szilagyi (Sipka, Boldogh and Szilagyi, 1977). They demonstrated that a rabbit MIF-containing supernatant could induce a macrophage disappearance reaction (MDR) when injected into the peritoneal cavity of *Rana esculenta*. The MDR is thought to be an *in vivo* manifestation of MIF activity; increasing the adherence of phagocytic cells to the peritoneal membranes, and thereafter reducing the number of cells that can be washed from the peritoneum (Adelman, Hammond, Cohen and Dvorak, 1979). The rabbit supernatant, however, only caused a 30% reduction in cell numbers in the *Rana* peritoneal cavity, this compares rather unfavourably with the 90% reductions routinely observed in mammalian experiments. Other workers have failed to confirm such findings for other lymphokines.

Thus although the physico-chemical properties of chicken chemotactic factor (CF) were very similar to human CF, chicken CF was not active in attracting human or guinea pig leucocytes, nor were human or guinea pig CF chemotactic for chicken cells (Altman and Kirchner, 1974). In addition, studies by Schauenstein and co-workers have shown that a chicken, thymic cell growth factor (TCGF)-containing supernatant had no effect on murine lymphoblast proliferation, and a potent mouse TCGF also had no effect on chicken lymphocytes (Schauenstein, Globerson, and Wick, 1982). The phylogenetic restriction, or lack of restriction, of other lymphokines must be investigated before the significance of these findings is clarified.

Apart from reports, by various groups, of migration inhibition reactions in representative species of reptile, anuran and urodele amphibian, and teleost and holostean fish (see Chapter Three of this thesis), only the studies of Le Clerc and co-workers describe lymphokine release in a non-mammalian animal. Using the echinoderm, Asterias rubens, they have demonstrated and characterized a factor, produced by mitogen stimulated axial organ cells, which causes an increase in the proliferation of non-stimulated axial organ cells (Brillouet, Luquet and Le Clerc, 1981). This mitogenic material they have termed "lymphokine-like substance". Prendergast and Susuki have also reported a protein extract of Asterias fordesi coelomocytes which, on injection, causes aggregation of the coelomocytes circulating in the injected portion of the animal. This extract, when injected intradermally into rabbits, mice, sheep, rats and guinea pigs, produced an erythematous, indurated lesion which was indistinguishable, by histology at least, from a typical delayed type hypersensitivity reaction (Prendergast and Susuki, 1970). Different fractions of the coelomocyte extract

were compared for their effects on guinea pig PEC migration, migration inhibitory activity was found in fractions containing y' protein of MW 32,000 Daltons. The same "sea star factor" (SSF) was also capable of activating mouse macrophages and was chemotactic for lymphocytes and macrophages (Willenborg and Prendergast, 1974) (Prendergast and Liu, 1976). It is tempting to speculate that the sea star factors may be the evolutionary precursors of vertebrate lymphokines, but great caution must be exercised in interpreting such findings, until the molecular biology of both can be determined and compared.

Despite the paucity of direct evidence for lymphokines in most animal phyla, it is possible to construct a theoretical scheme for their evolutionary development. Waksman has proposed that lymphokines arose from simple shed recognition units, or products of cell injury (Waksman, 1979). In primitive metazoan animals, such products may have served to guide phagocytic cells to sites of tissue damage. The evolution of cells which could recognise antigens, enabled a degree of specificity to be added to the release of these products, serving to integrate the recognition of antigen by one cell, with the effector functions of the macrophage. It seems probable that mediators such as MIF, MAF, CF and interferon which all act to modulate macrophage movement or activation, had their origins at this level. A non-lymphoid origin for these mediators is suggested by the fact that in mammals, MIF, CF and interferon have cytokine counterparts (Bigazzi, 1979) with very similar properties. The crucial importance that macrophages have in all aspects of immunity (Varesio, Landolfo, Giovarelli and Forni, 1980) has ensured that mediators which affect their function are conserved in evolution. In this light, the cross-

class activity of MIF is not surprising.

In the first level of lymphokine evolution involved the antigen-guided release of the simple inflammatory mediators from 'T'-like leucocytes, then the second level coincided with the evolution of 'T' and 'B' lymphocytes. 'T' cell recognition of antigens needed to be integrated with both 'T' and 'B' cell effector functions, the development of regulatory lymphokines with amplificatory and suppressive function, for example the interleukin series of mediators (Aarden, 1979) (Immunological Reviews, Vol.63) and IDS and SIRS (see Chapter Five) may have accomplished this.

The final level of lymphokine development accompanied the further sub-division of lymphocytes, which is at its most complex in the mammals. Thus a range of antigen specific helper and suppressor factors, which may carry determinants coded for in the major histocompatibility complex, have been demonstrated (Feldmann, Howie and Kontiainen, 1979), (Waltenbaugh, 1979).

Returning to the theme outlined in the introductory chapter, the Anura have long been known to display parallel in vivo cell mediated and humoral immune reactions, to those of mammals. These reactions are known, in mammals, to require co-operation between 'T' cells, macrophages and 'B' cells. Evidence that lymphokines are involved in these in vivo processes is limited, however, reports that lymphokine preparations injected intra-dermally, can cause delayed type hypersensitivity reactions (Yoshida and Cohen, 1974), and that anti-lymphokine antiserum can abrogate the normal DTH reaction, if injected with the test antigen (Yoshida, Bigazzi and Cohen, 1975), strongly support

such a role. The invivo functions of individual mediators are still subject to speculation, but their in vitro effects may reflect similar ones in vivo. Fig.6.2 outlines the possible in vivo roles of the lymphokines which have been described here for Rana, in processes such as graft rejection or DTH responses. Thus, following contact with antigen, a lymphocyte can release CFs which attract phagocytic cells; once in the proximity of the site of challenge, these phagocytes are immobilized by MIFs and also activated to increase phagocytosis and intracellular killing. Increased proliferation of other lymphocytes is caused by MF; these stimulated lymphocytes in turn secrete more inflammatory lymphokines. Once the antigenic challenge has been neutralized by the influx of immune cells, then IDS serves to damp down the proliferation, and MStF to free the macrophages from their immobile state. The dangers inherent in such simplification were revealed in Chapter Three where the possible helper and suppressor functions of MIF and MStF were discussed, the actual in vivo significance of mediators may not necessarily be related to their functions in an in vitro assay. Once again, purification of Rana lymphokines, and the development of in vivo assays will be required before these issues can be resolved.

The work presented in this thesis, has provided the first evidence for a range of lymphokines in an amphibian. Further work is required to fully elucidate the full range and physicochemical and physiological properties of these factors. The amphibia provide a suitable species to extend this work, with the clawed toad, Xenopus laevis. This species offers a number of advantages not enjoyed by Rana (Katagiri, 1978); it is easier to thymectomize, enabling investigations on the 'T' cell dependence of lymphokine

production and the role of lymphokines in 'T' cell development, to be performed; also, a number of cloned lines are now available, which should enable the production of MHC-restricted helper and suppressor factors, to be examined.

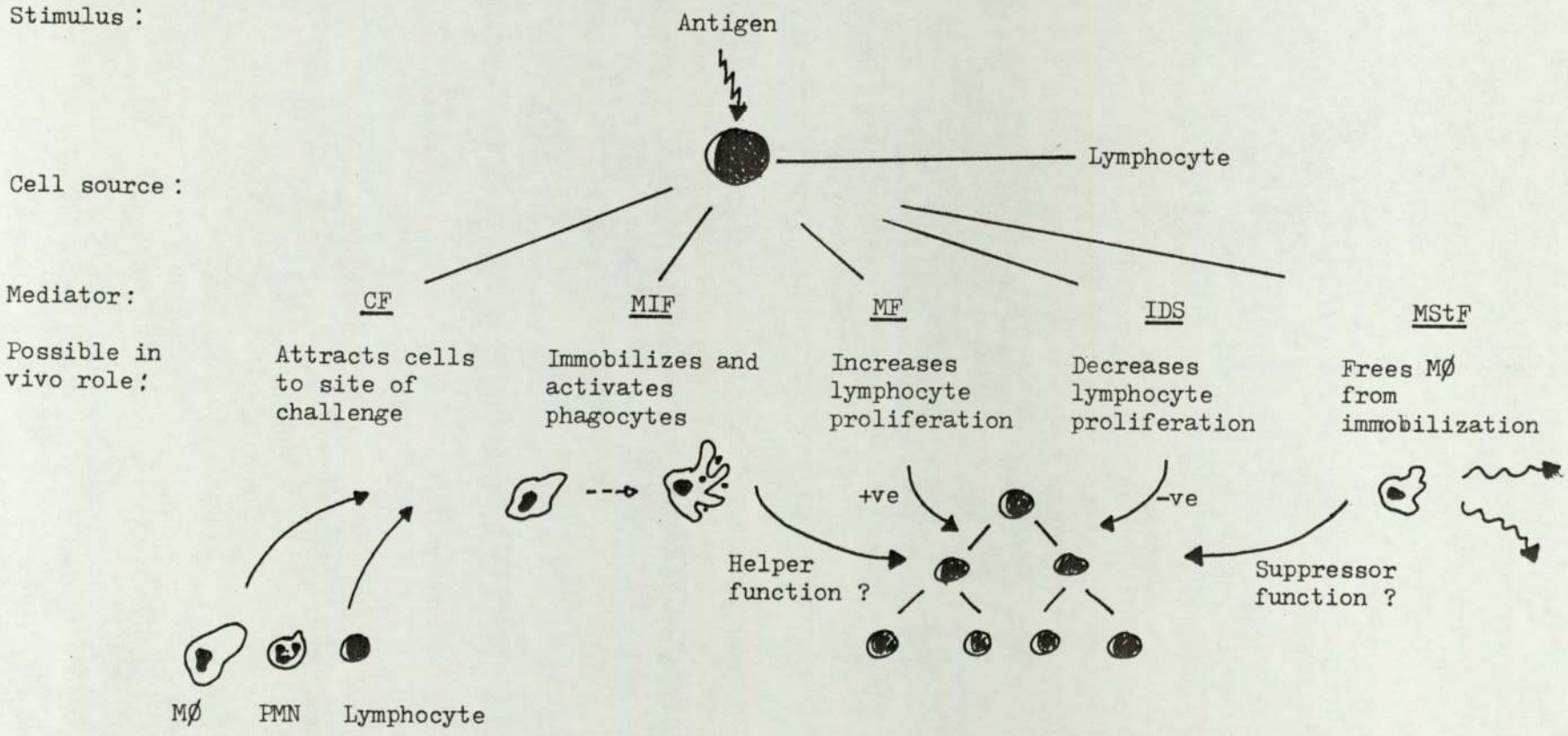
The lower order of complexity of the Xenopus MHC makes it a very viable system in which to study such genetically restricted factors, and their role in the regulation of the immune response. The comparison between amphibian and mammalian lymphokines, may also provide a route to identifying the existence of a "mother factor". The demonstration of extensive structural homologies, if found, would serve to confirm its existence. The immunopharmacological implications of such a structure, from which lymphokines with different target cell specificities and function could be synthesised, are enormous. Research on amphibian lymphokines is clearly at an early, yet very exciting stage, with great potential for future development.

Fig.6.1 SUMMARY OF RANA TEMPORARIA LYMPHOKINES AND THEIR IN VITRO PROPERTIES

Assay System	Mammalian Mediator	Ag/Mitogen induction	Soluble	MW range K Dalton	Sugar inhibition	Antibody mediated
Inhibition of PEC migration	MIF	Both	+	9-50	+ (fucose)	-
Stimulation of PEC migration	MStF	Ag	NT	NT	NT	NT
Stimulation of PEC glucose uptake	MAF	Mitogen	+	16-50	NT	-
Stimulation of directional PEC migration	CF	Both	+	16-27	NT	-
Stimulation of spleen cell proliferation	LMF	Both	+	16-50	NT	-
Inhibition of spleen cell proliferation	IDS	Mitogen	+	9-16 >50	NT	-

Fig. 6.2 Outline of possible in vivo functions of the lymphokines described for Rana temporaria.

Abbreviations in appendix.



APPENDIX List of abbreviations employed in this thesis

AEF	Allogeneic effect factor
BF	Blastogenic factor
CF	Chemotactic factor
con A	Concanavalin A
DTH	Delayed type hypersensitivity
HF	Helper factor
If	Interferon
IDS	Inhibitor of DNA synthesis
LMF	Lymphocyte mitogenic factor
LPS	Lipopolysaccharide
MAF	Macrophage activating factor
MEF	Migration enhancement factor
MI	Migration inhibition
MIF	Migration inhibition factor
MStF	Migration stimulatory factor
MØ	Macrophage
NK	Natural killer cell
PEC	Peritoneal exudate cell
PHA	Phytohaemagglutinin
PMN	Polymorphonuclear leucocyte
SF	Suppressor factor
SIRS	Soluble immune response suppresor
SSF	Sea star factor
T_{cyt}	Cytotoxic T cell
T_h	Helper T cell
T_s	Suppressor T cell
^3HT	Tritiated thymidine

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