STUDIES ON POLYMORPHONUCLEAR CELL MIGRATION UNDER AGAROSE AND THE MACROPHAGE CELL ADHERENCE.

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I declare that this thesis hereby submitted to the University of the North by me for the degree of Master of Science has not been submitted by me for a degree at another university and that it is my own original work.

Signed .....

A. GALBRAITH

Dated 15/6/82

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SUMMARY

This thesis is the study of (1) The agarose plate method for determining the migration of polymorphonuclear leucocytes and the effect of leucocyte inhibiting factor (LIF) on this migration. (2) The production of LIF and macrophage activating factor (MAF) from stimulated lymphocytes and (3) The effect of MAF on the adherence of macrophage cells to plastic surfaces.

The optimum conditions for migration were determined using criteria such as temperature, pH, type of agarose used, incubation time and conditions of incubation. The effect of plant mitogens on migration was also studied.

By pulsing lymphocytes with phytohaemagglutinin, concanavalin A or pokeweed mitogen and then incubating the lymphocytes, LIF was produced in the culture medium. The optimum time for incubation was studied as well as using varying quantities of the mitogens and pulsing for varying times. The activity of LIF produced was measured using the agarose plate method. It is suggested that there may be more than one LIF produced from stimulated lymphocytes and this is dependent on the mitogen used. The assay was found to be very sensitive and owing to its sensitivity, it may not be suitable for quantitative studies on LIF production, but would be suitable for determining abnormalities in their production.

The mixed lymphocyte reaction was studied and it was found that the LIF or LIF's produced in this reaction were in all probability different from those produced by mitogen stimulation. This was confirmed by gel filtration of the supernatants from lymphocytes stimulated by both methods.

A semi-automatic method of determining macrophage adherence is also described. This procedure employs the use of plastic tubes on whose walls, macrophage cells adhere. The cells remaining in the supernatant after incubation, are counted using a Sysmex microcell counter. This procedure is shown to be useful in determining the presence of macrophage activating factors (MAF), which are produced from lymphocytes upon stimulation with mitogenic substances.

An attempt was made to separate LIF from MAF from the supernatant of PHA pulsed lymphocytes, using Sephadex G100. It was found that using this system, MAF activity could be separated from LIF, but not vice versa and it is suggested that more than one MAF is produced from stimulated lymphocytes. With the mixed lymphocyte reaction, LIF could be completely separated from MAF activity and again more than one MAF was produced.

OPSOMMING

Hierdie tesis is 'n studie van (1) Die agaroos-plaat metode vir die bepaling van die migrasie van polimorfe-nukleere leukosiete en die effek van die leukosiete inhibisiefaktor (LIF) op hierdie migrasie. (2) Die produksie van LIF en die makrofaag aktiverende faktor (MAF) van gestimuleerde limfosiete en (3) Die effek van MAF op die adhesie van makrofaag-selle op plastiek oppervlaktes.

Die optimum kondisies van migrasie is bepaal deur gebruik te maak van standaarde soos temperatuur, pH, tipe agaroos-plaat, inkubasie periode en kondisies van inkubasie. Die effek van plant-mitogeen op migrasie is ook bestudeer.

Deur polsing van limfosiete met fitohemaglutinien, konkanavalin A en 'pokeweed' mitogeen en die inkubasie van die limfosiete, is LIF geproduseer in die kultuur medium. Die optimum tyd vir inkubasie is bestudeer, asook die gebruik van verskillende hoeveelhede van die mitogeen en polsing vir verskillende periodes. Die LIF geproduseerd is gemeet met die gebruik van die agaroos-plaat metode. Dit is voorgestel dat daar waarskynlik meer as een LIF deur die gestimuleerde limfosiete geproduseer word en dat dit afhanklik is van die mitogeen wat gebruik is. Dit is gevind dat die toets baie sensitief is en dus waarskynlik nie geskik is vir die kwantitatiewe studies van LIF produksie nie maar wel geskik is om abnormaliteite in hulle produksie vas te stel.

Die gemengde limfosiete reaksie is bestudeer en dit is bevind dat die LIF of LIF's geproduseer in hierdie reaksie in alle waarskynlikheid verskil van die geproduseer deur mitogeenstimulasie.

'n Semi-outomatiese metode om makrofaag adhesie te bepaal is ook beskryf. Hierdie prosedure maak gebruik van plastiek buise waarin die makrofaagselle aan die wande vaskleef. Die oorblywende selle in die supernatent na inkubasie is met behulp van 'n Sysmex mikroselteller bepaal. Hierdie prosedure het getoon dat dit nuttig is vir die bepaling van die teenwoordigheid van makrofaag aktiverende faktore (MAF) wat geproduseer word uit limfosiete na stimulasie met mitogeniese stowwe.

'n Poging is gemaak om LIF van MAF te skei van die supernatent van PHA gepulseerde limfosiete deur gebruik te maak van Sephadex G100. Deur gebruik van hierdie metode is bevind dat MAF aktiwiteit geskei kan word van LIF, maar nie andersom nie. Dit is voorgestel dat meer as een MAF geproduseer is deur gestimuleerde limfosiete. Met die gemengde limfosiet reaksie kan LIF heeltemal geskei word van MAF aktiwiteit en is daar weer meer as een MAF geproduseer.

CONTENTS

GENERAL INTRODUCTION

Lymphokines are soluble non-antibody products of lymphocyte activation by specific antigen and by some non-specific antigens.

Although lymphokines act as molecular mediators of the cellular immune response (Waksman, 1978), many lymphokines have been shown to be produced by both T- and B-lymphocytes (Chess et al., 1975). But as lymphokines are mainly part of the cell mediated immune response, they are produced mainly by the T-lymphocytes (Ling & Kay, 1975).

Some of the lymphokines which have already been characterised are as follows:

1. Dumonde et al., (1968) reported a lymphocyte mitogenic factor which enhances lymphocyte DNA synthesis.

2. Kolb et al., (1968) reported lymphocyte cytotoxic factors which enhance lymphocyte cytotoxicity in vitro eg they can cause cytolysis of chicken erythrocytes and are called lymphotoxins (Ruddle & Waksman, 1968)

3. Lolekha et al., (1970) reported macrophage agglutinating factor which enhances macrophage aggregration both in vivo and in vitro.

4. Dekaris et al., (1971) reported macrophage spreading factor which inhibits the spreading of macrophages on glass cover slips.

5. Interferons have also been included in the lymphokine group of substances (Glasgow, 1966 & Green et al., 1970).

6. Several growth inhibitors or cytostatic factors have been reported (Holtzman et al., 1973), one of which is called colony inhibiting factor as it inhibits the growth of HeLa cells in culture.

7. Green et al., (1970) reported a proliferation inhibitory factor which prevents the growth of human cells in culture.

8. Macrophage migration inhibition factor (MIF), was found by David, (1966) and Bloom & Bennet, (1966), to be produced by activated lymphocytes, thirty four years after the macrophage migration inhibition phenomenon <u>in vitro</u> was described by Rich & Lewis, (1932).

9. Mooney & Wakson, (1970) reported macrophage activating factor which increased the adherability of macrophages on culture dishes.

10. Rocklin, (1974) described leucocyte migration inhibitory factor (LIF) which inhibits the migration <u>in vitro</u> of polymorphonuclear cells.

At least fifty biologically active factors have been described as lymphokines on the basis of biological studies (Waksman, 1978) and it is believed that the production of these substances from T-lymphocytes helps control cell mediated immunity in animals (Waksman, 1978). It has been shown that there is a cooperation between T-lymphocytes and B-lymphocytes (Miller et al., 1968) which is mediated by a specific lymphokine.

Until all the lymphokines so far named in the literature have been isolated and their function determined, it may be that some named lymphokines are identical with others. For example it was long thought that macrophage activating factor was distinct from macrophage migration inhibitory factor, but it appears that they are the same glycoproteins (Nathan et al., 1973). Leucocyte migration inhibitory factor which was thought to be identical to macrophage activating factor was shown by Rocklin, (1974) to be distinct.

This thesis is a study of the effects of two factors produced from stimulated lymphocytes, namely leucocyte migration inhibiting factor (LIF) and macrophage activating factor (MAF). As these lymphokines are chemically distinct and serve different functions in cell mediated immunity (Rocklin, 1974), this thesis is divided into three parts:

Part one deals with the production and measurement of

LIF, part two deals with the production and measurement of MAF and part three deals with the separation of MAF from LIF by gel filtration to try and confirm that they are separate substances with different functions.

PART 1.

LEUCOCYTE MIGRATION INHIBITORY FACTOR

Introduction

A. Methods of Measuring LIF.

The leucocyte migration inhibition test has been accepted as an *in vitro* correlate of cell mediated immunity in man (George & Vaughan, 1964 ; Soborg & Bendixen, 1967). The test can be used to determine to which antigens a host has been sensitized , to provide the possible explanations for the mechanism of tissue damage in certain diseases eg rheumatoid arthritis and elucidate the mechanisms of certain immunodeficiency states (Snyderman et al., 1977). The first reliable method of determining migration of cells was introduced by George and Vaughan (1962) who placed the polymorphonuclear cells in capillary tubes and observed the effects of a test material on their migration out of the tube.

Bloom & Bennet, (1966) used this method to study the inhibition of migration of guinea pig macrophage cells. This work showed the existence of MAF. Søborg & Bendixen, (1967) used the method to study the migration of human peripheral leucocytes. Carpenter et al., (1968) developed a method where cells could be shown to migrate under an agar gel. This method was further modified by Clausen, (1971) and it is Clausen's method, using agarose gel that is now used by many workers to determine the migration of many cell types and the effect of various substances

on this migration. (This method has also been developed to show chemotaxis. Orr & Ward, (1978) showed the migration of leucocytes towards various chemotactic reagents placed in adjacent wells to the cells).

There are two basic variations of the agarose plate method to determine LIF activity (Willoughby et al., 1978).

1. The direct or one-stage technique in which the buffy coat cells are mixed with the stimulating mitogen or antigen, before being added to wells cut in agarose plates. During incubation for 24 hours, LIF is produced by the lymphocytes and inhibits the migration from the wells of the target granulocytes in the same cell mixture (Willoughby et al., 1978). The mitogen could of course have an effect on the migration of the cells and this would have to be taken into account. This was studied in this thesis.

2. The indirect or two-stage technique in which separated lymphocytes are first cultured with the selected stimulant. The cell-free supernatants from these cultures containing LIF (as well as a number of other lymphokines) are then mixed with target granulocytes in the second stage to measure the inhibition of cell migration from wells in agarose plates.

The indirect method is more time consuming but enables

the reaction to be separated into two components, viz: LIF production by lymphocytes and LIF action on granulocytes and more readily allows standardization of the procedure (Willoughby et al., 1978). The first method is not suitable to determine the action of mitogens on lymphocytes as they themselves inhibit migration (Lomnitzer, 1977).

In this thesis, the indirect method is used exclusively for measurements of LIF production and activity. However as migration assays have limited clinical usefulness because of the complexity of performing relatively few tests and reproducibility remains a major point of contention in migration assays (Glasser & Fiederlein, 1979), the experimental work is in part an attempt to look into the variables which could affect the assay. An attempt is made to determine just how reliable the LIF assay is.

B. Production of LIF.

The production of lymphokines from lymphocytes by mitogenic stimulation is as yet a fairly non-specific process as the mitogens do not act upon one population of lymphocytes specifically (Ling & Kay, 1975). It is hoped that in the future mitogens will be discovered or tailored which will activate defined sub-populations of lymphocytes. For instance different mitogens do not behave

identically in the way they affect lymphocyte proliferation and metabolism (Ling & Kay, 1975). It is possible to separate the two major types of lymphocytes, T- and B-cells by E and EAC rosette formation, as T-lymphocytes form rosettes with sheep erythrocytes under different conditions from the B-lymphocytes and the rosettes being of a larger size than the non-rosetting cells, separation can be obtained (Cooper & Bain, 1971 ; Pretlow & Luberoff, 1973). Using this method, T- and B-cell populations can be stimulated separately.

There is evidence that methods of purification of lymphocytes modify cells (Cooper & Rubin, 1965 ; Oppenheim et al., 1966). It is debatable whether it is better to study separated T- and B-lymphocytes or whether to study mixed T- and B-lymphocytes (the proportion of each which can be known), using specific mitogens for each cell type if they are available. In this study, mixed T- and B-lymphocyte populations were used.

C. Mitogenic Stimulation of Lymphocytes.

Lymphocytes of most species are activated by the Phaseolus Vulgaris mitogen, phytohaemagglutinin (PHA), by other lectins eg pokeweed mitogen (PWM) and concanavalin A (Con A).

These mitogens have been called 'non-specific' stimulants because a high level of response is obtained with blood or tissue lymphocytes of most individuals with no requirement for prior sensitisation to the substance concerned.

For this study, the above three plant lectins were used. A great disadvantage of these compounds is that their purity varies considerably from different suppliers. Eg: PHA from Wellcome Research Laboratories seems to bind more tightly, and to a larger number of receptors on the lymphocyte surface, than that obtained from Difco Laboratories (Kay, 1967 ; Skoog et al., 1974). Therefore it is important, when stimulating lymphocytes with mitogens to use mitogens from the same supplier.

Con A is isolated from the jack bean (Canavalia ensiformis) and along with PHA activates mainly the T-lymphocytes (Janossy et al., 1973 ; Davies et al., 1971). Whereas PWM from Phytolacca Americana activates both B- and T-lymphocytes (Goldschneider & Cogen, 1973 ; Weber, 1973).

Various other classes of compounds have been used to stimulate lymphocytes and it appears that dextran sulphate (Ruhl et al., 1974), tuberculin purified protein derivative (PPD) (Sultzer & Nilsson, 1972) and some lipopolysaccharide endotoxins (Greaves & Janossy, 1972)

are specific for B-lymphocytes. Therefore as well as the lectins already discussed, some experimental work was done on the action of Escherichia coli lipopolysaccharide on the production of lymphokines.

When lymphocytes from the blood of two unrelated individuals with different HLA tissue types are mixed together and cultured for several days, both sets of cells are stimulated by each other in a similar way to the action of mitogens ie both populations acting as 'stimulator'and 'responder' cells. This is known as the mixed lymphocyte reaction (MLR).(Bain et al., 1964).

It is possible to arrest the growth of the stimulator cells by mitomycin C treatment (Bach & Voynow, 1966) or radiation (Kasakura & Lowenstein, 1968) and therefore one can study the production of lymphokines from the responder cells. The consequence of the MLR is important in histocompatability testing and in transplantation (Dauset et al., 1970 ; Rychlikova et al., 1971). Because of this a comparison of the MLR with lectin stimulation of lymphocytes was carried out to see whether similar lymphokines, namely LIF and MAF are produced.

SURVEY OF METHODOLOGY

1. Separation of Lymphocytes.

Blood contains many differing types of cells, therefore it is important to obtain as pure a population of lymphocytes as possible for study. In order to separate blood cells, use is made of the fact that lymphocytes tend to be of a lesser density than the granulocytes and erythrocytes. Employing a density gradient centrifugation technique, it is a relatively simple matter to prepare a fairly pure lymphocyte population.

Originally an albumin gradient technique was used (Vallee et al., 1947), but this method has the disadvantage in that unless the blood is defibrinated, platelets will contaminate the lymphocyte fraction (Shortman & Szenberg, 1969). Also factors other than density, notably pH and osmolarity affect the standardisation of this technique (Ling & Kay, 1975).

Gorcznski et al., (1970) introduced a method using Ficoll (a polysucrose of mol. wt. 400 000), instead of albumin. This method has the advantage that Ficoll is uncharged and unlike albumin does not bind ions which may alter the osmolarity of the separating medium. Ficoll tends to clump cells, so a dispersing agent is required, as well as buffering compounds to keep the pH at about 5,5. Unfortunately this method has proved difficult to

standardise (Gorczynski et al., 1971 ; Ling & Kay, 1975).

Boyum, (1968) introduced a method using Ficoll in conjunction with a balanced solution of the sodium, calcium and magnesium salts of metrizoic acid (3-acetamido-2,6,6 triiodo-5-N-methyl acetamido benzoic acid), which is sold under the trade name of Hypaque as an intravenous contrast media. Boyum used defibrinated blood in his original method but this step is not necessary (Senyk & Hadley, 1976 ; Lavergne & Harrington, 1978). The method of Boyum is the most widely used in which whole heparinised blood can be employed without any pretreatment and the lymphocytes can be separated into a fraction, relatively free from polymorphonuclear cells but containing monocytes and suitable for subsequent culture. Occasionally a bad separation may be produced which may be due to differing blood compositions and cell density but this is unusual except in the case of blood from patients with certain types of liver disease. (Eg Histopaque manufactured by Sigma Chemical Corporation seems to separate lymphocytes better than Ficoll-Paque, made by Pharmacia*).

2. Lymphocyte Culture.

Lymphocytes are not very demanding cells as far as growth is concerned and a simple balanced salt solution

* M. Alberts Personal Communication.

supplemented with serum will allow some cell growth (Ling & Kay, 1975). In most cultures of lymphocytes, the cells are being activated and therefore need a fully supplemented medium to realise maximum activity. Most conventional synthetic media when supplemented with 5-20% serum, support the growth of activated lymphocytes (Paul, 1965). A stable pH of 7,4 is important in culture and as bicarbonate is usually present, as a buffering substance, it is the balanced salt base of the media. This can cause alkalisation of media during growth, unless carbon dioxide is present in the gas phase (Ling & Kay, 1975). Williamson & Cox (1968) and Darzynkiewicz & Jacobson (1971) replaced the bicarbonate buffer with Hepes (N-2-hydroxy methyl-piperazine-N-2-ethane sulphonic acid) which did away with the need for carbon dioxide in the gas phase.

Most available media need to be supplemented with glutamine.

To prevent as far as possible, bacterial and fungal contamination of the media, it is advisable to add streptomycin, penicillin and amphotericin. Penicillin and streptomycin can be substituted with gentamycin (Armstrong, 1973).

3. Preparation of Polymorphonuclear Cells.

As the presence of lymphocytes together with polymorphonuclear cells (PMN's) inhibits the migration of the PMN's (Glasser & Fiederlein, 1979), it follows that one cannot use the erythrocyte sedimentation method

for the preparation of PMN's with or without the addition of dextran. As is mentioned above, lymphocytes are first removed by density gradient centrifugation, leaving the erythrocytes and granulocytes in the same fraction. The erythrocytes can then be sedimented by gravity. The addition of dextran speeds up this process (Ling & Kay, 1975). What is probably more convenient is to lyse the erythrocytes in an hypotonic solution as it is much faster than the above technique and does not appear to significantly harm the leucocytes (Glasser & Fiederlein, 1979). Erythrocytes can be lysed by using distilled water by flash lysis (Naylor & Little, 1975). This takes 15 seconds and timing is critical, otherwise the leucocytes will be damaged (Glasser & Fiederlein, 1979). Saponin can be used as a lysing agent (Cutts, 1970), but as this compound lyses by disruption of the cell membranes, it is not surprising that leucocyte death with this procedure is high and it is therefore not a very suitable agent (Glasser & Fiederlein, 1979). The most widely used lysing agent is ammonium chloride, a compound which can freely cross leucocyte membranes and does not appear to harm the leucocytes (Boyle, 1968 ; Glasser & Fiederlein, 1979).

4. Agarose Plate Migration.

Migration inhibition assays have proved to be useful for testing leucocyte sensitisation against a variety of antigens. As has been stated already, it is often difficult to achieve good reproducibility and accuracy

and this may considerably affect the validity of the results (Glasser & Fiederlein, 1979). Many technical modifications have been described (Clausen, 1971 ; Scheetz et al., 1972 ; Houck & Chang, 1973 ; Harrington & Stastney, 1973). The method used in the experimental work to follow is the method used by Clausen with some variations which are described. Most workers using this method, determine the migration index (MI) by measuring the area of migration of the leucocytes in both the control and test samples, using planimetry and/or projection microscopy (De Halleux & Deckers, 1975 ; Willoughby et al., 1978 ; Palit et al., 1978). However, assuming even migration, the area should be proportional to the diameter or radius. The following expression has been used:

$$MI = \frac{\text{DIAMETER OR RADIUS OF MIGRATION OF TEST CELLS}}{\text{DIAMETER OR RADIUS OF MIGRATION OF CONTROL CELLS}}$$

(Borkowsky & Lawrence, 1979). This last method does not involve expensive equipment, so is the method of choice in the practical work to follow. Jokipii & Jokipii,(1974), compared the migration areas of the dense centre and of the whole fan using the capillary tube technique and found either to be accurate. As it is usual for the dense area of migration to be fairly uniform compared to the whole area of migration (See Fig. 1.),

the diameter or radius of the dense area should prove to be more accurate in measuring the MI.

METHODS

When aseptic techniques were used, the work was carried out as far as possible in a laminar flow cabinet.

1. Specimen Collection

Whole blood was collected by venepuncture and transferred to a heparinised sterile specimen container. The heparin used must be preservative free. The type of heparin used does not seem to affect the cells (Glasser & Fiederlein, 1979) but the presence of preservatives, for example phenol, does (Rosenberg & David, 1970).

As far as could be ascertained, the blood was collected from healthy black and white donors. A record was kept of the race of the donor, for comparison. The blood was processed as soon as possible after collection which was never more than one hour, except in one experiment where the effect of storage on the cells was studied.

2. Preparation of Lymphocytes

This was essentially the method used by Boyum (1968) -- Ficoll-Hypaque was prepared by dissolving 90g of Ficoll in 1 litre of distilled water (heating with stirring is required). 200ml of Hypaque-65 and approximately 200ml of distilled water are then added to bring the density to 1,077. This was checked using a hydrometer. The prepared solution was put into 250ml screw top bottles and the level carefully marked. The bottles were

sterilised by autoclaving and after cooling to room temperature, the solutions were made up to the mark using sterile distilled water to restore the original density. The resulting solution was stored at $4^{o}C$. Before use it was brought to room temperature.

Phosphate buffered saline (PBS) pH 7,4 was prepared from 8,5g sodium chloride, 1,28g disodium hydrogen phosphate, 0,156g sodium dihydrogen phosphate and made up to 1 litre with distilled water. The pH was adjusted to 7,4 using either 0,1M sodium hydroxide or 0,1M hydrochloric acid depending upon the initial pH. 200 000 units penicillin G (Crystapen) and 100mg of streptomycin sulphate (Novostrep Solution) were added and the solution filtered under pressure through a 2,2u membrane filter for sterilisation. The PBS was left at room temperature.

Eagle's minimum essential medium (MEM) was used single strength with the addition of 2,2g sodium bicarbonate, 4,53g hydroxyethyl piperazine ethane sulphonic acid (Hepes) per litre and antibiotics as for the PBS. The pH was adjusted to 7,4. After sterilisation as for PBS 2ug/ml of sterile amphotericin B (Fungizone injection) was added aseptically. The medium was kept at $4^{o}C$ for not more than six weeks.

15ml of Ficoll-Hypaque was put into a 50ml conical plastic tube and 20ml of heparinised blood carefully

layered over it by angled pouring. The resulting double layer was centrifuged at 400g for twenty five minutes at room temperature. This produced a density gradient centrifugation and the lymphocytes were found at the interface of the plasma and Ficoll-Hypaque layer. The plasma was removed carefully using a sterile Pasteur pipette and the mononuclear cell layer was transferred aseptically into another 50ml conical tube. The mononuclear cells were washed twice with 20ml PBS and once with 20ml MEM, using a ten minute, 200g centrifugation between each step. After the final wash, the cells were resuspended in 5ml MEM and a 0,8ml aliquot removed aseptically for counting and E- and EAC-rosette formation. The cells were counted using a Coulter counter after haemolysis of any red cells that may be present.

3. Viability of Lymphocytes.

This was checked on a small aliquot of the cells, using a 1% solution of trypan blue and checked for dye exclusion from the live cells as opposed to the staining of the dead cells.

4. Lymphocyte Culture.

There was some variation in the methods for the cultivation of lymphocytes but the standard method is given here and any variation is mentioned in the experimental section.

Culture was carried out in $25cm^2$ culture flasks at $37^{o}C$ in an atmosphere of 5% carbon dioxide (CO_2). The lymphocytes were suspended at a concentration of 1×10^6 cells per ml in MEM with 4,5ml per culture flask. The MEM was supplemented with 0,05ml of a sterile 0,2M glutamine solution (This solution is stored at $-20^{o}C$) and 0,5ml of pooled human AB serum. This gives a final concentration of glutamine 20uM and 10% with respect to the serum.

The cells were stimulated with varying concentrations of phytohaemagglutinin (PHA), concanavillin (Con A) or pokeweed mitogen (PWM), using different conditions. (See experimental section)

Incubation time was usually for sixty hours. After incubation, lymphocyte viability was checked using trypan blue and the suspension centrifuged at 1500g for five minutes. The supernatant was then stored at $-20^{o}C$.

5. Preparation of Polymorphonuclear Cells.

Medium 199 (M 199) was prepared as for MEM. After removal of the lymphocytes, the tubes were spun at 1000g for five minutes to ensure all cells would be found in the lower layer. The Ficoll-Hypaque was then removed by aspiration and the lower layer washed into a conical flask with a solution of ammonium chloride 0,83%, potassium hydrogen carbonate

0,2% and ethylene diamine tetraacetic acid (EDTA) disodium salt 0,06% at $4^{o}C$*

The resulting mixture was kept on ice until lysis of the erythrocytes was complete. Usually this took not more than ten minutes. Many workers use only 0,83% ammonium chloride (Federlin et al., 1971 ; Bullen & Losowsky, 1977 ; Kedar et al., 1976).

But by using the above method, it was found lysis sometimes took over one hour. Lysis with distilled water can also be used (Hakansson & Venge, 1980 ; Willoughby et al., 1978), but as this lyses leucocytes in under five minutes (Olling et al., 1979), it is better to use ammonium chloride solution, as it does not affect leucocyte viability and migration (Glasser & Fiederlein, 1979).

After lysis, the remaining suspension was centrifuged at 300g for five minutes, followed by two washes in PBS and one with M199. The PMN's from different donors were normally combined during the washing step to give a heterologous PMN population. The PMN's were suspended in M199 at a concentration of 2 X 10^{5}cells per 10ul.

6. Agarose Plate Migration.

TC Medium 199 was prepared as a 10 times concentrate, 10g Medium 199 was dissolved in 100ml distilled water,

* A. Rabson Personal Communication.

penicillin and streptomycin were added as for MEM. The solution was filter sterilised using a pressure syringe and was kept for up to six months at 4°C with no loss of LIF activity as far as leucocyte migration was concerned.

Agarose plates were prepared by dissolving 240mg of agarose in 30ml of sterile distilled water. Heating at 95°C for thirty minutes was needed for solvation. The solution was then cooled to 50°C in a water bath before the addition of a mixture of 3ml Medium 199, 10 X concentrate, 3ml horse serum and 5 drops of sterile 10% sodium bicarbonate solution. The resulting mixture was immediately pipetted in 7ml aliquots to plastic Petri dishes (Diameter 6cms) and left to set for at least one hour at room temperature. The plates were used the same day as they were prepared.

There was sometimes a variation in the above method. This will be described in the experimental section where appropriate.

The actual method of migration was subjected to a considerable variation of times, number of polymorphonuclear cells used and volume of test substance ie lymphokine (LIF) present in supernatant or lectin used. Conditions and time of migration will be mentioned in the experimental section and in the discussion.

Wells were cut in the agarose plate, using a 2,2mm steel tube attached to a vacuum pump. The control well ie the well with no MIF or lectin was left as far as possible from the test wells, so as to minimise any chemotaxis that might result. The cell suspension was added to the wells using a micropipette (NB This stage of the experimental work was not aseptic).

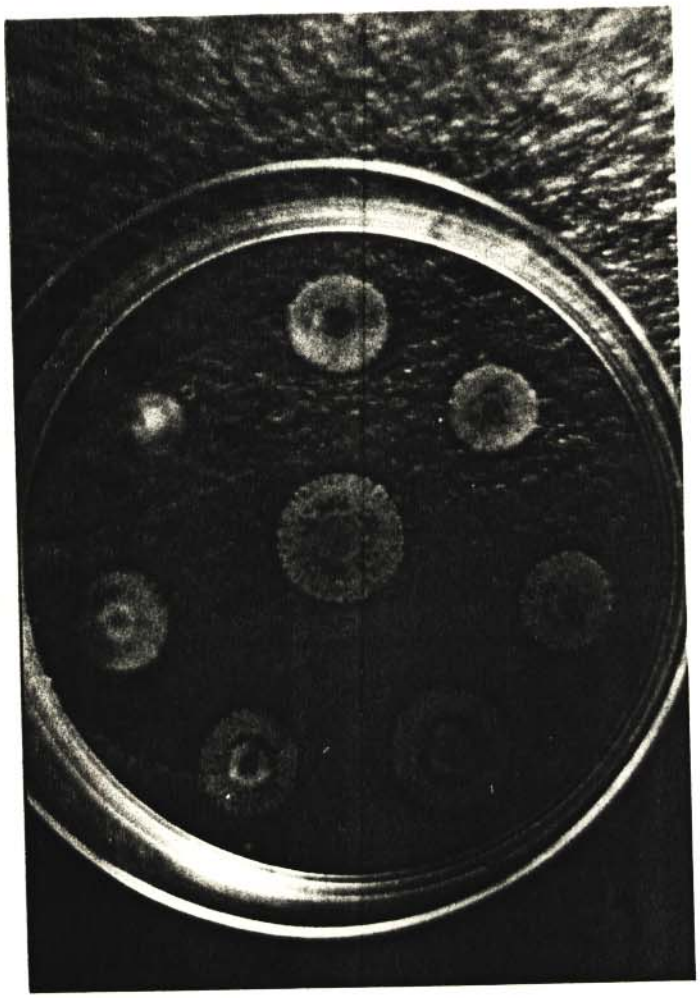
Incubation was in a 5% CO_2 atmosphere at 37°C for 20 hours.

After incubation, the plates were flooded with methanol and left for fifteen minutes and the methanol decanted. 40% formaldehyde solution was added for fifteen minutes to fix the cells to the plastic. The agarose was removed by gently hooking a spatula under the edge of the gel which then fell cleanly out of the dish. The cells were stained with either Giemsa or May & Grunwald's stain for five minutes and washed in running water, when the area of migration could be seen visually. (See Plate 1.)
Most of the time, the migration area was annular, if not, the plate was discarded. The radius of the migration area was measured using a Visio-Pan microscope, giving a magnification of 20X. (The actual migration is therefore multiplied by a factor of 20). The radius was taken from the edge of the well up to the

distinctly stained edge of the migration area as scattered cells were usually found far out from the distinct area. (See Fig 1.)

Plate 1.

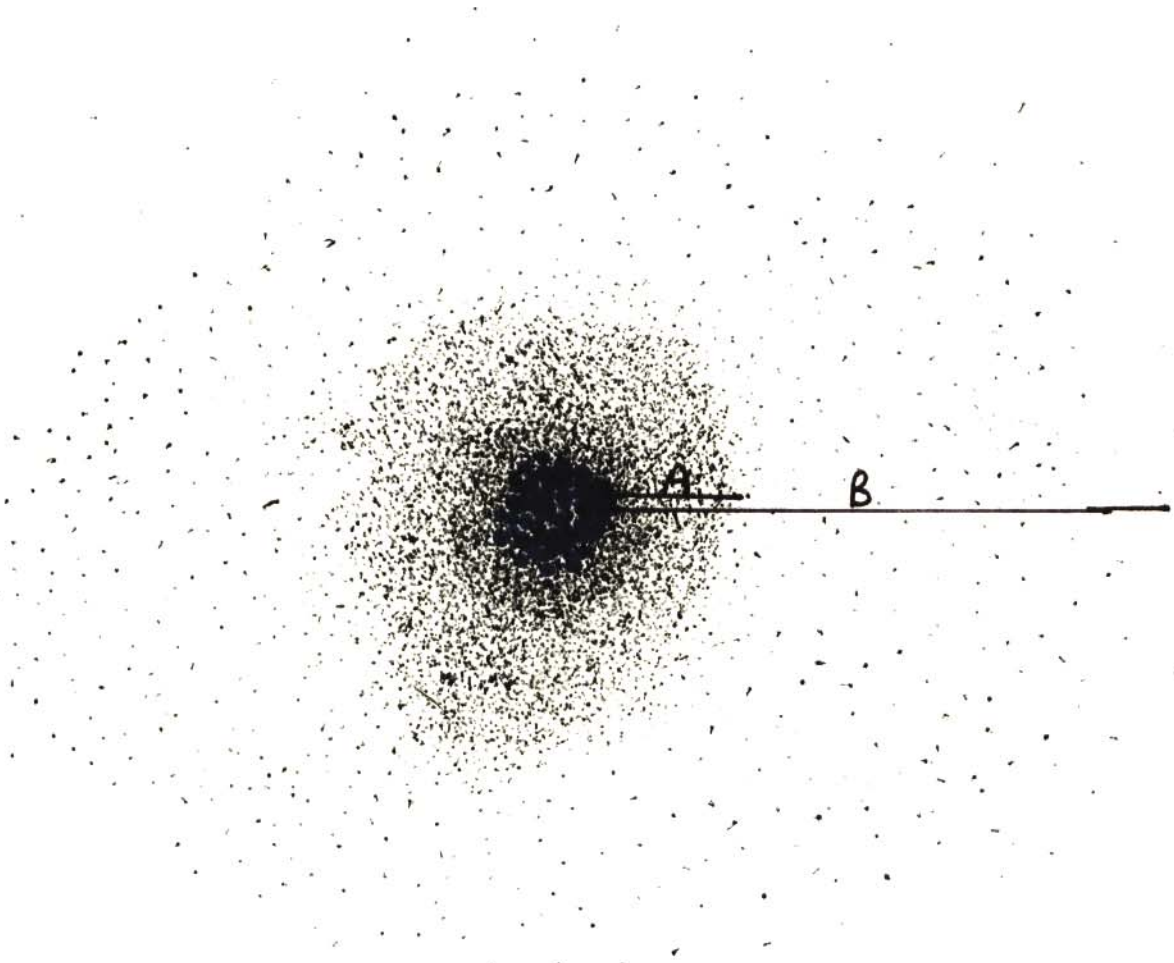
Photograph Showing PMN Migration After Staining.



The dense area of cells is only visible on this photograph. The area of cell scattering as shown in Fig 1., is only visible on magnification.

Fig 1.

Diagram of Migration of PMN Cells under Agarose.



Radius A is the radius of the dense area of migration. This area is not exactly annular and the radius was measured at the widest part of the migration, which was never more than 0,5cm at 20X magnification, greater, than the shortest radius. Radius B shows the maximum migration of cells and as can be seen, this area is much less annular than the inner area. The difference in radius between the centre and the peripheral cells was often 2 to 3cm at 20X magnification.

MATERIALS

Agarose Type 2	Sigma Chemical Corporation
Concanavillin Type iv	Sigma Chemical Corporation
Crystapen Injection	Glaxo
Disposable Ware	Falcon Plastics
Eagles Medium	Wellcome
Ficoll	Pharmacia
Foetal Calf Serum	Flow Laboratories
Fungizone Injection	Squibb
Glutamine	Merck Chemicals
Guinea Pig Complement	Wellcome
Haemolysing Reagent	Harleco
Heparin Preservative Free	Glaxo
Horse Serum	Flow Laboratories
Hypaque	Sterling Winthrope
Membrane Filters	Millipore
Novostrep Injection 1g/3ml	Novo
Phytohaemagluttinin Type V	Sigma Chemical Corporation
Pokeweed Mitogen	Sigma Chemical Corporation
Rabbit Haemolytic Serum	Wellcome
Specimen Containers	Sterilin
TC Medium 199	Wellcome
Trypan Blue	British Drug House
Visio-Pan Microscope	Reichart

All other chemicals were analytical grade.

EXPERIMENTAL SECTION AND RESULTS

SECTION 1.

Standardisation of the Method.

In this section no attempt was made to inhibit the migration of the PMN cells, but to study the effect of various parameters on it.

1. Assessment of Agarose from Different Suppliers.

Agarose plates were set up as described previously, using six different types of agarose.

These were:

a. Agarose — BDH Chemicals
b. Agarose — Behring
c. Agarose — Miles Laboratories
d. Agarose Type 1. — Sigma Chemical Corporation.
e. Agarose Type 2. — Sigma Chemical Corporation.
f. Agarose Type 3. — Sigma Chemical Corporation.

2×10^5 PMN cells from the same donors, in 5ul of MEM were added to the wells. (NB. The Behring agarose did not produce a firm gel which proved difficult to make the wells in). The experiment was done in triplicate with three wells to each plate and incubated for 15 hours at $37^{\circ}C$.

Results are tabulated in Table 1.

TABLE 1.

Agarose Plate	Radius of Migratory Area (cm)			Mean
	i	ii	iii	
1 a	5,0	5,0	5,5	
2 a	5,0	5,0	5,0	
3 a	5,5	5,0	5,0	5,1
1 b	3,0	2,0	1,0	
2 b	2,0	3,0	3,0	
3 b	3,0	2,0	1,0	2,2
1 c	5,0	5,0	5,0	
2 c	5,5	5,0	5,5	
3 c	5,0	5,5	5,0	5,2
1 d	7,0	7,0	8,0	
2 d	8,0	7,0	7,5	
3 d	7,5	7,0	7,0	7,3
1 e	10,0	11,0	10,0	
2 e	11,0	10,0	11,0	
3 e	10,0	11,0	11,0	10,6
1 f	6,0	7,0	7,5	
2 f	7,5	6,0	7,5	
3 f	7,5	6,0	7,0	6,9

These results show the variation in migration using different types of agarose and show the advantage of using the Sigma Type 2, as it gives the maximum migration and was used in all the following experiments.

2. Assessment of Different Sera.

Agarose plates were set up, as previously described, using varying types of sera in their preparation.

a. Gibco Foetal Calf Serum.
b. Difco Foetal Calf Serum.
c. Flow Foetal Calf Serum
d. Miles Foetal Calf Serum.
e. Human AB Serum (Northern Transvaal Blood Transfusion Service).
f. Flow Foetal Calf Serum. (Inactivated)
g. Flow Horse Serum.
h. Miles Horse Serum.

The Miles sera were all kept at $4^{o}C$, the rest were stored at $-20^{o}C$. The experiment was done in duplicate, with three wells to each plate.
No. of cells, volume and time of incubation were as for Experiment 1.
Results are tabulated in Table 2.

TABLE 2.

Agarose Plate	Radius of Migratory Area (cm)			Mean
	i	ii	iii	
1 a	10,0	11,0	11,5	
2 a	11,0	9,5	11,0	10,7
1 b	10,5	10,5	11,0	
2 b	11,0	11,0	11,0	10,8
1 c	10,0	10,5	11,0	
2 c	9,5	9,5	10,0	10,1
1 d	10,5	10,5	11,0	
2 d	10,5	11,0	11,0	10,8
1 e	9,0	9,0	8,5	
2 e	8,5	9,5	9,5	9,0
1 f	8,0	8,5	9,0	
2 f	9,5	8,5	8,5	8,7
1 g	5,0	6,0	5,5	
2 g	6,5	6,5	6,5	6,0
1 h	9,5	10,0	10,5	
2 h	9,5	9,5	10,0	9,8

These results show that there is little difference in the migration of the cells using different types of serum, except for the heat inactivated foetal calf serum. Although horse serum did not produce the best migration of the cells, because of its cheapness compared to the other types, the Miles type was used in the subsequent experiments.

3. Effect of pH and the Addition of HEPES.

The pH of the liquid agarose solution was measured quickly at about 42°C, before gelling took place, the electrode being kept at 40°C in distilled water to prevent gelling at the tip. The pH of the gel may have been slightly higher at the incubation temperature of 37°C, but this was ignored for practical purposes. The pH was adjusted using either 1% sodium carbonate solution or 0,1M hydrochloric acid.

The migration was done in duplicate with three wells to each plate. Number of cells, volume and time of incubation as for Experiment 1.

Results are shown in Table 3. and Figure 2. HEPES was used at varying concentrations instead of sodium bicarbonate and together with sodium bicarbonate. The results are shown in Table 3a. The pH was kept at 7,4.

TABLE 3.

pH	Radius of Migratory Area			Mean
	i	ii	iii	
6,5	5,5	5,5	5,0	
	5,5	5,0	5,5	5,3
6,7	6,0	6,0	6,0	
	6,0	6,0	6,0	6,0
6,9	6,0	6,0	6,0	
	6,5	5,5	6,0	6,0
7,1	6,5	7,0	6,5	
	7,0	6,5	6,5	6,7
7,3	8,5	8,5	9,0	
	9,0	8,5	9,0	8,8
7,4	9,5	8,5	9,5	
	8,5	9,0	9,0	9,0
7,5	8,5	9,0	8,5	
	8,5	9,0	9,0	8,8
7,6	8,5	8,5	8,5	
	8,5	9,0	8,5	8,6
7,8	8,0	8,5	8,0	
	8,0	8,0	8,0	8,1
8,0	8,0	7,5	7,5	
	7,5	7,5	7,5	7,6

The pH optimum of the migration of the PMN's under agarose is between pH 7,3 and 7,5, as shown by these results.

Increase or decrease in the pH from these values, results in an inhibition of the migratory process.

All the following experiments were carried out at pH 7,4.

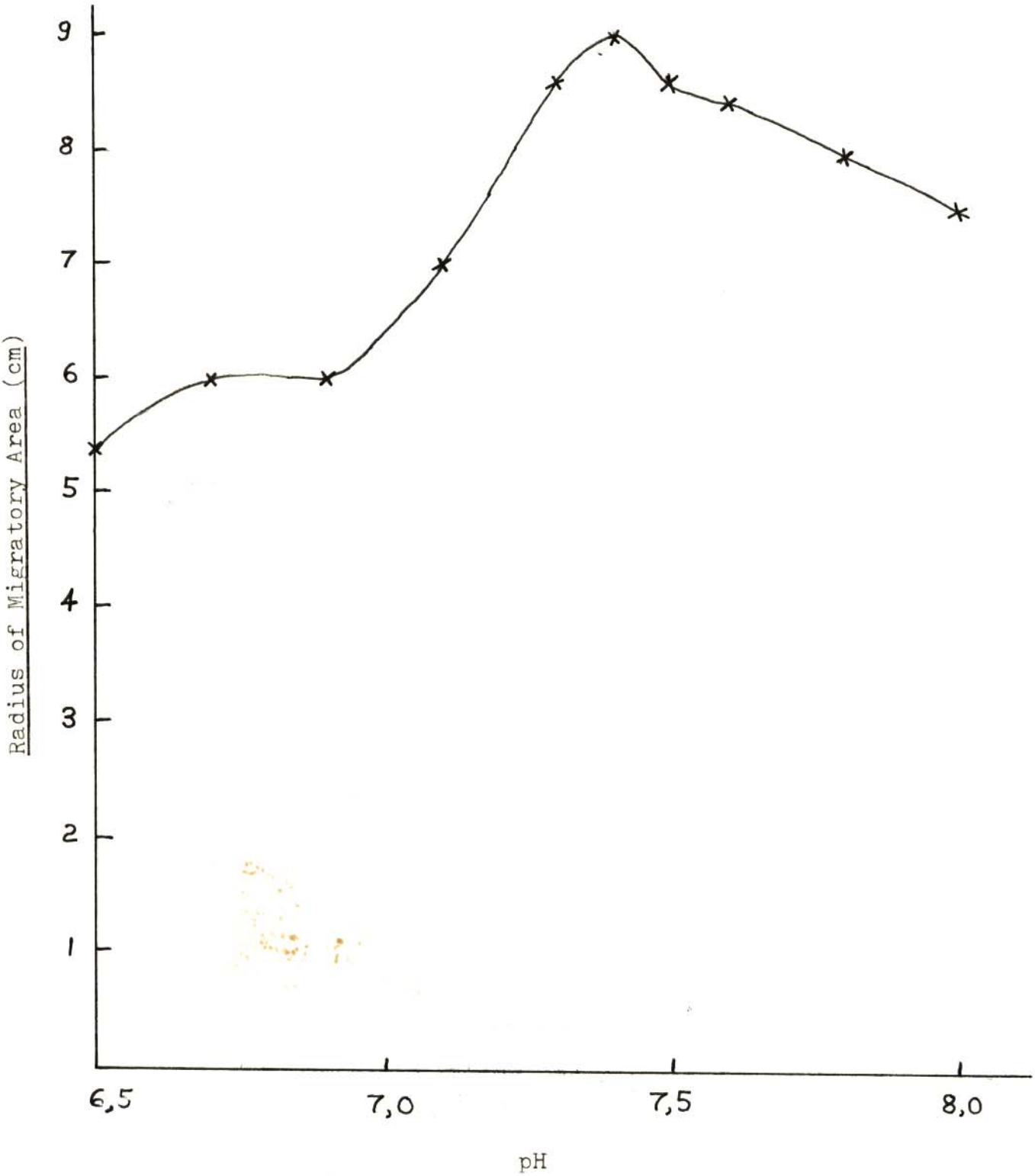
TABLE 3a.

Concentration of HEPES Not Containing $NaHCO_3$	Radius of Migratory Area			Mean
	i	ii	iii	
4 mM	3,0	2,0	2,0	2,3
5 mM	3,0	3,0	3,0	3,0
6 mM	3,0	2,0	2,0	2,3

Concentration of HEPES With $NaHCO_3$	Radius of Migratory Area			Mean
	i	ii	iii	
4 mM	3,0	2,0	3,0	2,7
5 mM	2,0	2,0	2,5	2,2
6 mM	2,0	2,0	2,0	2,0

The presence of HEPES in the agarose, shows a significant inhibition of the migratory process at pH 7,4, whether sodium bicarbonate is present or not.

Fig 2.



4. Effect of Varying Plate Incubation Time.

In this experiment, the duration of the incubation of the plates with PMN's in the wells was varied in order to determine the optimum time to carry out the fixation of the cells. Number of cells and volumes were as for Experiment 1. The results are shown in Table 4.

TABLE 4.

Duration of Incubation	Radius of Migratory Area			Mean
	i	ii	iii	
30 Mins.	0	0	0	0
	0	0	0	0
1 Hour	0	0	0	0
	0	0	0	0
2 Hours	1,0	1,0	1,0	
	1,0	1,0	1,0	1,0
3 Hours	1,5	1,5	1,5	
	1,5	1,5	1,5	1,5
4 Hours	2,0	1,5	1,5	
	2,0	2,0	2,0	1,8
5 Hours	2,5	2,5	2,5	
	3,0	3,0	3,0	2,8
15 Hours	10,0	11,0	11,0	
	9,5	11,0	10,5	10,5
20 Hours	11,5	11,0	11,5	
	11,0	11,0	10,5	11,1
24 Hours	10,0	11,5	12,0	
	12,5	12,0	11,0	11,5

The twenty and twenty four hour plates were difficult to measure due to excess scattering at the periphery. Incubation for five hours and less produced minimal migration of the cells. Fifteen hours incubation which is a convenient time for incubation (ie overnight), produced an easily measurable migration and this time was used in all experiments excluding this one.

5. Effect of Temperature and Carbon Dioxide.

Prepared plates were incubated for 15 hours at varying temperatures and conditions as shown in Table 5.

The experiment was carried out in duplicate with two wells per plate.

Number of cells and volume were as for Experiment 1.

The results are shown in Table 5.

TABLE 5.

Temperature		5%CO_2	Radius of Migration		Mean
			i	ii	
20°C	1a	Present	3,0	3,5	
	2a	Present	3,5	3,0	3,3
25°C	1b	Present	4,0	4,0	
	2b	Present	4,0	4,0	4,0
30°C	1c	Present	7,0	7,5	
	2c	Present	6,5	6,0	6,8
37°C	1d	Present	9,0	9,5	
	2d	Present	9,5	9,5	9,4
37°C	1e	Absent	0	0	
	2e	Absent	0	0	0

The presence of CO_2 is essential for migration to occur and the temperature optimun is 37°. Lower temperatures produce a decrease in migration.

All the following migrations were carried out at 37°C.

6. Effect of Erythrocyte Contamination.

The erythrocyte count after one haemolysing step with ammonium chloride was very variable and the results tabulated, are taken at random from a series of several experiments to obtain a progressive increase in the percentage of erythrocytes present during the migration. The number of erythrocytes are shown as a percentage of the total cell count. This was carried out as in Experiment 1., using 1 X 10^5 PMN's in 5ul. To decrease the percentage of red cells, a second haemolysing step was carried out in some cases for five minutes in ammonium chloride solution.

After this step, no erythrocytes were left as far as could be determined using a Coulter counter. The results are tabulated in Table 6.

TABLE 6.

Percentage Erythrocytes	Radius of Migratory Area	
	One Haemolysing Step	Two Haemolysing Steps
23	9	-
35	8	-
51	7	-
60	6	-
68	9	-
72	9	9,5
81	9	9,0
87	10	11,5
91	7	10,5

The presence of erythrocytes is not inhibitory to migration until at least 90% of the total cells present are erythrocytes when an inhibition of migration does occur. The haemolysing steps do not affect the migratory process and in the following experiments, if more than 88% of the cells were erythrocytes, a second haemolysing step was carried out.

7. Effect of PMN Number and Volume of Suspension.

The wells in the agarose plates could hold a maximum of 10ul. Therefore, in this experiment, the effect of using either 5ul or 10ul of cell suspension was looked at. During the course of the same experiment, the number of cells in each well was varied.

The experiment was carried out in duplicate with two wells per plate.

The results are shown in Table 7.

TABLE 7.

Number of PMN's	Volume	Radius of Migratory Area		Mean	S.D.
1×10^5	5ul	4,0	4,0		
1×10^5	5ul	5,0	5,5	4,6	0,75
2×10^5	5ul	14,5	13,0		
2×10^5	5ul	14,0	13,0	13,6	0,75
4×10^5	5ul	9,5	7,0		
4×10^5	5ul	8,0	9,5	8,5	1,20
6×10^5	5ul	10,0	8,0		
6×10^5	5ul	9,5	8,0	8,9	1,00
8×10^5	5ul	5,5	4,5		
8×10^5	5ul	4,0	4,0	4,5	0,71
1×10^6	5ul	5,5	4,0		
1×10^6	5ul	4,5	5,5	4,9	0,75
2×10^5	10ul	14,0	14,0		
2×10^5	10ul	14,5	14,5	14,3	0,29
4×10^5	10ul	8,0	8,0		
4×10^5	10ul	8,0	8,0	8,0	0,00
6×10^5	10ul	8,5	8,0		
6×10^5	10ul	8,5	8,3	8,3	0,29

The 5ul volumes of PMN suspension, when used in the wells give similar radii of migration to the 10ul volumes but the variation is higher using the 5ul volumes especially when many cells are used.

The 10ul volumes containing 2 X 10^5 cells produced a large migration and a low standard deviation is used in the subsequent experiments.

8. Effect of Using PMN Cells from One or Several Donors.

In this experiment, the cells used were either from one donor or a mixture of PMN's from two to four different donors.

In all the experiments carried out so far, PMN cells were mixed, usually from four donors, to minimise individual variations in PMN characteristics. To see if this was justified, a series of experiments to ascertain this, were carried out. As only four bloods were taken each day, the experiment was repeated on two other occasions.

2×10^5 PMN's were used per well in 10ul of MEM. Results are shown in Table 8.

Table 8.

Donor	Radius of Migratory Area	Donor Cells	Radius of Migratory Area
A	6,0		
B	9,0	A+B	9,0
C	10,0	A+B+C	10,0
D	12,0	A+B+C+D	12,0
E	12,0		
F	12,0	E+F	12,0
G	9,0	E+F+G	12,0
H	6,0	E+F+G+H	12,0
I	9,0		
J	12,0	I+J	10,0
K	12,0	I+J+K	13,0
L	9,0	I+J+K+L	13,0

There is a considerable variation in migration, when using single donor PMN's in the wells. This variation is much less marked when mixed donor cells are used.

9. Effect of Time and Temperature on Plate Storage.

In this experiment, agarose plates were prepared as normal, and stored at 4°C, room temperature (20°C), or 37°C for one to three days. Migrations were carried out as before, using, as well as the stored plates, freshly prepared ones. The experiment was carried out in triplicate, with three wells to each plate.

The results are shown in Table 9.

TABLE 9.

Age of Plate & Storage Condition		Radius of Migratory Area a.	b.	c.	Mean
Fresh	1.	13,0	13	12	
	2.	12,5	11	11,5	
	3.	12,0	13	12,5	12,3
4° 1 Day	1.	13,0	13,5	11,0	
	2.	11,5	11,0	11,0	
	3.	11,5	12,5	13,5	12,1
RT 1 Day	1.	12,5	13,5	12,0	
	2.	11,5	12,0	13,0	
	3.	12,0	13,0	11,0	12,3
37° 1 Day	1.	0	0	0	
	2.	0	0	0	
	3.	0	0	0	0
Fresh	1.	13,5	11,5	10,0	
	2.	10,5	10,5	11,0	
	3.	12,5	11,0	11,0	11,3
4° 2 Days	1.	8,0	8,5	9,5	
	2.	9,5	8,0	9,5	
	3.	9,0	9,0	9,0	9,0
RT 2 Days	1.	5,0	5,0	4,5	
	2.	0	4,5	6,0	
	3.	4,5	5,5	4,0	4,3
Fresh	1.	11,0	10,0	11,5	
	2.	12,0	12,5	11,5	
	3.	11,0	11,0	11,0	11,3

TABLE 9 (Continued).

Age of Plate & Storage Condition		Radius of Migratory Area a.	b.	c.	Mean
4° 3 Days	1.	4,0	4,5	6,0	
	2.	7,5	4,5	6,0	
	3.	6,0	6,0	6,0	5,6
RT 3 Days	1.	0	0	0	
	2.	0	0	0	
	3.	0	0	0	0

Prepared agarose plates give better migration of PMN's, if they are kept either at room temperature or at 4°C for no longer than one day. Further storage is detrimental to migration, as is storage at 37°C. All future experiments were carried out using freshly prepared plates as were all previous experiments.

10. Effect of Lectins.

In this experiment, the effect of lectins on migration was examined. In one case, the effect of pulsing the cells with one of the lectins for 2 hours and then washing the cells three times, before adding them to the agarose plate wells was examined. Secondly, the lectin was added to the PMN cells and the mixture added to the wells. Untreated cells were also added as controls to separate wells on the same plate. Lectins were used in various concentrations and the three lectins used were: Con A, PWM and PHA.

The results for Con A are shown in Table 11A.
The results for PWM are shown in Table 11B
The results for PHA are shown in Table 11C.
The experiments were done in duplicate with two test wells and two standard wells per plate.
All other parameters were as usual.
The results are given as Migratory Indices.

TABLE 11A.

Con A in Well	Radius of Migratory Area				M.I.		Mean M.I.
ug/ml	Test		Control				
	a	b	a	b	a	b	
0,22	12,0	12,0	12,5	11,5	0,97	1,00	
	12,0	11,0	11,0	11,0	1,1	1,00	1,00
0,44	10,5	11,0	10,5	11,5	1,00	0,96	
	11,5	12,0	12,0	12,5	0,96	0,96	0,97
0,88	11,5	12,0	12,0	9,5	0,96	1,20	
	10,5	10,5	10,5	10,5	1,00	1,00	1,00
1,76	11,0	11,5	11,5	11,0	0,96	1,10	
	11,0	11,0	11,5	11,0	0,96	1,00	1,00
3,13	11,5	12,5	11,5	11,5	1,00	0,92	
	12,5	12,0	12,0	12,0	1,00	1,00	0,98
6,25	11,5	11,5	12,0	12,0	0,96	0,96	
	11,0	11,0	9,5	10,5	1,20	1,10	1,10
12,5	5,0	5.50	8,5	11,0	0,59	0,50	
	5,5	5,5	12,0	12,0	0,46	0,46	0,50
25	6,5	6,5	12,0	11,0	0,54	0,59	
	7,0	7,0	11,0	11,5	0,64	0,61	0,60
50	10,5	10,0	12,5	12,0	0,84	0,83	
	10,5	11,0	13,0	11,5	0,81	0,96	0,86
100	0	0	12,0	12,0	0	0	
	0	0	12,0	13,0	0	0	0

TABLE 11A (Continued).

Con A in Well	Radius of Migratory Area				M.I.		Mean M.I.
ug/ml	Test		Control				
	a	b	a	b	a	b	
Pulsed Cells							
25	11,5	11,5	11,5	12,0	1,00	0,96	
	11,0	11,5	11,5	11,0	0,96	1,10	1,00
50	10,5	10,0	12,0	10,0	0,88	1,00	
	10,5	11,0	12,5	11,5	0,84	0,96	0,92
100	0	0	12,0	12,0	0	0	
	0	0	12,0	13,0	0	0	0

Con A affects migration at concentrations of 12,5ug/ml and above. The results show an increase in migration up to 100ug/ml.

Cells pulsed with Con A at 100ug/ml still show complete inhibition of migration but at 50ug/ml and below, little or no effect on migration is produced.

TABLE 11B.

PWM in Well	Radius of Migratory Area				M.I.		Mean M.I.
ug/ml	Test		Control				
	a	b	a	b	a	b	
1	4,0	4,0	12,0	12,0	0,33	0,33	
	4,0	4,0	12,0	12,0	0,33	0,33	0,33
2	4,0	4,0	12,0	12,5	0,33	0,32	
	4,0	4,5	11,5	11,0	0,35	0,41	0,39
3	4,5	4,5	12,0	11,0	0,38	0,36	
	4,0	4,0	10,5	11,0	0,38	0,36	0,38
4	4,0	4,5	11,0	10,5	0,36	0,43	
	4,0	4,0	11,0	9,5	0,36	0,42	0,39
5	4,5	4,5	11,0	11,0	0,41	0,41	
	4,0	4,0	12,5	12,5	0,32	0,32	0,37
10	2,5	2,0	11,0	11,0	0,23	0,18	
	2,5	2,0	11,5	10,5	0,22	0,19	0,21
20	1,0	1,0	12,0	12,0	0,08	0,08	
	1,0	1,0	12,0	11,0	0,08	0,09	0,08
25	1,0	1,0	12,5	11,0	0,08	0,09	
	1,0	1,0	10,5	12,0	0,1	0,08	0,09
30	1.0	1,0	11,0	12,5	0,09	0,08	
	1,0	1,0	11,0	11,0	0,09	0,09	0,09

TABLE 11B (Continued).

PWM in Well ug/ml	Radius of Migratory Area				M.I.		Mean M.I.
	Test		Control				
	a	b	a	b	a	b	
Pulsed Cells							
1	10,0	10,0	11,0	11,0	0,91	0,91	
	10,5	10,5	12,0	11,5	0,88	0,91	0,9
5	10,0	10,5	11,0	10,5	0,91	1,0	
	10,0	10,0	11,0	10,5	0,91	0,95	0,94
10	11,0	11,0	11,0	12,0	1,0	0,92	
	10,5	10,0	12,5	11,0	0,84	0,91	0,92
15	10,0	10,0	12,0	12,0	0,92	0,92	
	10,5	11,0	11,5	11,0	0,91	1,0	0,94

PWM, when present in the wells with the PMN's, inhibits migration strongly at concentrations of as low as 1ug/ml and at concentrations of 15ug/ml and above, migration is almost inhibited completely. Pulsing the PMN's with PWM has little effect on the migration.

TABLE 11C

PHA in Well	Radius of Migratory Area				M.I.		Mean M.I.
ug/ml	Test		Control				
	a	b	a	b	a	b	
1	8,0	7,5	12,0	11,0	0,67	0,68	
	8,0	8,0	11,5	11,5	0,7	0,7	0,69
2	8,5	8,0	10,5	11,0	0,81	0,73	
	8,0	8,0	11,0	12,0	0,73	0,67	0,74
3	7,0	7,0	12,5	11,0	0,56	0,64	
	7,5	8,0	11,5	11,0	0,65	0,73	0,65
4	8,0	7,0	10,5	10,5	0,76	0,67	
	7,5	7,0	11,0	10,5	0,68	0,67	0,7
5	7,0	6,5	11,0	11,0	0,64	0,59	
	7,0	7,0	12,0	11,5	0,58	0,61	0,61
10	6,0	6,0	10,0	9,5	0,6	0,63	
	6,5	6,0	10,0	9,5	0,65	0,63	0,63
15	6,0	5,5	10,0	11,0	0,6	0,5	
	5,0	5,5	11,0	11,5	0,46	0,48	0,51
20	5,0	5,0	12,0	11,5	0,48	0,43	
	5,0	5,0	11,0	10,0	0,46	0,5	0,45
25	5,0	5,0	10,0	11,5	0,5	0,44	
	4,5	5,0	10,5	9,5	0,43	0,53	0,48
30	4,5	5,0	11,5	12,0	0,39	0,42	
	4,5	4,5	12,5	11,0	0,36	0,41	0,40

PHA, when present in the wells at concentrations varying from 1ug/ml to 30ug/ml gives gradual inhibition of migration as the concentration is increased.

TABLE 11C (Continued).

PHA in Well	Radius of Migratory Area				M.I.		Mean M.I.
Pulsed Cells							
ug/ml	Test		Control				
	a	b	a	b	a	b	
1	10,5	11,0	12,0	12,5	0,88	0,88	
	10,5	10,5	12,0	12,0	0,88	0,88	0,88
5	10,5	11,0	11,5	12,0	0,88	0,88	
	9,5	9,5	12,0	11,5	0,79	0,83	0,84
10	9,5	9,0	9,5	11,0	1,0	0,82	
	9,5	9,0	11,5	11,5	0,83	0,78	0,86
20	9,5	9,0	12,0	12,0	0,79	0,75	
	9,0	9,0	12,0	11,5	0,75	0,78	0,77

Pulsing the cells with PHA, at low concentrations has a slight effect on migration but even at 20ug/ml concentration, is not markedly inhibited.

12.

1. The Effect of Lymphokines on PMN Migration.

2. The Effect of Culture Conditions on Lymphokine Production.

In order to establish a normal migratory index, a series of experiments was prepared, culturing lymphocytes from normal white and black donors, under the conditions already mentioned on Page 18. The cells were pulse stimulated with 5ug PHA per ml of culture media and 1 X 10^6 cells per ml.

Blood from 40 white and 65 black donors was used. In order to test for the presence of LIF, the supernatants were added to the PMN cells as follows, 0,1ml of supernatant was added to 0,9ml of a PMN cell suspension, containing 2 X 10^6 cells per ml. The cells were centrifuged for five minutes at 200g, the supernatant removed and the cells resuspended in 0,1ml of MEM. 10ul of this was added to the agarose plate wells and the plates incubated for 15 hours at $37^{o}C$ in a 5% CO_2 atmosphere.

The results are shown in Table 12A for lymphokines from lymphocytes obtained from black donors and 12B for those from lymphocytes obtained from white donors. The results of the radii are the mean of the three wells for both control and test samples.

The mean per cent T and B cells are also shown for both populations.*

* R. Mogashoa Personal Communication.

TABLE 12A

Radius of Migratory Area

Control	Test	M.I.
3,5	1,0	0,28
6,0	2,0	0,33
9,0	6,5	0,72
8,5	2,0	0,24
8,0	2,0	0,25
10,0	8,0	0,80
4,0	2,5	0,63
5,0	3,0	0,60
9,0	7,5	0,83
9,0	8,0	0,89
6,0	2,5	0,42
10,0	8,0	0,80
6,0	1,5	0,25
7,5	3,0	0,25
6,0	2,5	0,42
6,0	4,0	0,67
11,0	5,0	0,46
11,0	10,0	0,91
8,5	2,5	0,29
7,5	3,5	0,46
8,5	3,0	0,35
10,0	7,0	0,70
7,0	1,5	0,21

TABLE 12A (Continued).

Radius of Migratory Area

Control	Test	M.I.
5,0	1,5	0,30
10,0	8,0	0,80
8,0	5,0	0,63
6,5	4,0	0,62
5,5	4,5	0,82
6,5	4,0	0,62
7,5	6,0	0,80
8,5	7,5	0,88
7,0	3,0	0,43
8,0	1,5	0,19
10,0	8,0	0,80
9,0	2,5	0,27
8,0	3,0	0,38
8,5	3,5	0,41
6,0	4,0	0,67
7,0	3,5	0,50
7,5	7,0	0,93

Mean M.I. = 0,55 $\pm$ 0,24

Mean % Tcells = 67,2 $\pm$ 10,1

Mean % Bcells = 19,4 $\pm$ 3,9

Mean Radius of Control = 7,6 $\pm$ 1,8

Mean Radius of Test = 4,3 $\pm$ 2,4

TABLE 12B

Radius of Migratory Area

Control	Test	M.I.
6,5	4,5	0,69
6,5	4,5	0,69
8,0	3,0	0,38
7,5	3,0	0,40
8,5	3,5	0,41
6,0	5,0	0,83
12,0	10,0	0,83
7,0	4,0	0,57
5,0	2,0	0,40
6,0	4,5	0,75
6,0	3,5	0,58
5,0	2,5	0,50
6,0	1,5	0,25
9,0	4,5	0,50
9,0	6,5	0,72
2,0	1,5	0,75
6,0	4,5	0,75
10,0	4,5	0,45
11,0	3,0	0,27
8,0	5,5	0,69
11,0	9,0	0,82
11,0	7,0	0,64
11,0	9,0	0,82
5,5	2,0	0,36

TABLE 12B (Continued).

Radius of Migratory Area

Control	Test	M.I.
8,0	6,0	0,75
8,5	3,5	0,41
7,0	3,5	0,50
7,5	4,0	0,53
8,0	3,0	0,38
8,0	3,5	0,44
9,0	5,0	0,60
10,0	5,5	0,55
9,5	3,5	0,37
7,5	3,5	0,47
8,5	3,5	0,41
10,0	5,0	0,50
10,0	5,5	0,55
10,0	6,5	0,65
10,0	4,5	0,45
9,0	4,5	0,50
7,5	2,5	0,33
8,5	3,5	0,41
9,0	5,5	0,61
9,5	5,5	0,58
4,5	2,0	0,44
6,5	3,5	0,54
6,5	3,5	0,54
7,0	4,0	0,57

13. Assessment of the Effect of Lymphokine Containing Supernatant, Added to the PMN's.

Two lymphokine containing supernatants were added to the PMN's in varying volumes.

Both of the supernatants had been already examined under the conditions of Experiment 12, using 0,1ml of supernatant. One of the supernatants (A) had produced a low M.I. of 0,36 and the other (B) had produced a high M.I. of 0,83.
The supernatant was added in volumes from 10ul to 200ul to a suspension of 2 X 10^6 PMN's, to give a final volume of 1ml. The resulting mixture was treated the same as in Experiment 12. The results are shown in Table 13A and 13B and were obtained in duplicate with two test wells and two control wells per plate.

TABLE 13A

Volume of Supernatant	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
10ul	10,0	10,5	10,0	10,5	-	-	
	10,0	10,0	9,5	10,0	-	-	-
20ul	9,5	9,5	10,0	10,0	-	-	
	10,0	9,5	9,0	9,0	-	-	-
30ul	8,0	8,0	10,0	10,0	0,8	0,8	
	8,5	8,0	10,0	10,0	0,85	0,8	0,81
40ul	3,5	3,5	9,5	10,5	0,37	0,33	
	3,5	3,5	10,5	9,5	0,33	0,37	0,35
50ul	3,5	3,5	10,0	10,0	0,35	0,35	
	3,5	3,5	10,0	10,0	0,35	0,35	0,35
70ul	3,0	3,5	9,5	10,0	0,32	0,35	
	4,0	3,5	10,5	11,0	0,38	0,32	0,34
90ul	4,0	4,0	10,5	10,0	0,38	0,4	
	3,5	3,5	10,5	10,0	0,33	0,35	0,37
100ul	4,0	4,0	9,5	9,5	0,42	0,42	
	3,5	3,5	10,0	10,0	0,35	0,35	0,39
200ul	4,0	4,0	10,0	10,0	0,4	0,4	
	3,5	4,0	9,5	10,0	0,37	0,4	0,39

Increasing the amount of supernatant, containing LIF, added to the PMN cells does not affect the M.I. on the addition of very small amounts. At 20ul and below, no inhibition of migration takes place and at 30ul, only a very small inhibition of migration takes place.

TABLE 13B

Volume of Supernatant	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
10ul	10,0	10,0	11,0	10,5	-	-	
	10,5	10,5	10,5	10,0	-	-	-
20ul	10,5	10,5	10,5	10,5	-	-	
	11,0	10,0	10,0	10,0	-	-	-
30ul	9,5	10,0	10,5	10,0	-	-	
	9,5	10,0	10,0	10,0	-	-	-
40ul	9,5	10,0	10,0	10,0	-	-	
	9,5	10,0	9,5	9,5	-	-	-
50ul	10,0	10,0	10,0	9,5	-	-	-
	10,5	10,0	11,5	10,0	-	-	-
70ul	8,0	8,0	9,5	10,5	0,4	0,76	
	8,5	8,5	10,0	10,0	0,85	0,85	0,83
90ul	8,5	8,0	10,0	10,0	0,85	0,8	
	7,5	8,0	9,5	9,5	0,79	0,84	0,82
100ul	7,5	8,0	9,5	10,0	0,79	0,8	
	8,5	8,0	9,5	10,0	0,9	0,8	0,82
200ul	7,5	8,0	9,5	10,0	0,79	0,8	
	7,5	8,0	10,0	9,5	0,75	0,84	0,80

In this case, a volume of 70ul had to be added to produce inhibition of migration, but like Table 13A, further increases in volume, produced no difference in the M.I.

14. Effect of Storage, Freezing and Thawing on LIF.

The effect of storage on the LIF containing supernatants at -20°C was investigated. As power cuts are common at the University of the North, often for prolonged periods, supernatants were thawed and refrozen. Therefore, it was important to establish if this had any adverse effects on the LIF activity. Three random supernatants from normal donors were selected, LIF activity measured, the remainder frozen and thawed for four successive occasions. The LIF activity was measured after each thawing process. The results are shown in Table 14A. Another two samples were measured for LIF activity. The supernatants were divided into two portions and one portion was frozen for one month and the other for two months. After the times stated, the LIF activity was measured.

The results are shown in Table 14B.

TABLE 14A

	Radius of Migratory Area				M.I.		Mean
	Test		Control				
Sample 1.	a	b	a	b	a	b	
Fresh	3,0	3,5	9,5	9,5	0,32	0,37	0,34
1 Thawing	4,0	3,5	10,0	10,5	0,40	0,33	0,37
2 Thawings	4,0	4,5	10,0	10,0	0,40	0,45	0,43
3 Thawings	9,0	9,0	10,0	9,5	0,90	0,95	0,93
4 Thawings	10,5	10,0	10,0	10,0	-	-	-
Sample 2.							
Fresh	6,0	5,0	9,0	9,0	0,67	0,56	0,62
1 Thawing	6,5	5,0	9,0	9,5	0,72	0,53	0,63
2 Thawings	6,5	5,0	9,0	10,0	0,72	0,50	0,61
3 Thawings	8,5	8,5	9,5	10,0	0,9	0,85	0,88
4 Thawings	8,0	8,5	9,0	9,0	0,89	0,94	0,92
Sample 3.							
Fresh	6,0	5,5	10,5	11,5	0,57	0,49	0,52
1 Thawing	6,5	5,0	10,0	9,0	0,65	0,56	0,60
2 Thawings	6,5	7,0	11,0	10,5	0,59	0,67	0,63
3 Thawings	5,5	7,0	7,0	6,5	0,79	-	-
4 Thawings	8,5	9,0	9,0	9,0	-	-	-

The freezing and thawing processes have little or no effect on the capacity of the supernatant to produce inhibition of migration. Further thawing and freezing produces a decrease in this capacity.

TABLE 14B

Storage Time	Sample	Radius of Migratory Area				M.I.		Mean
		Test		Control				
		a	b	a	b	a	b	
Fresh	1	3,5	3,5	10,0	10,0	0,35	0,35	0,35
	2	6,0	6,0	9,5	10,0	0,63	0,6	0,62
1 Month	1	4,5	4,0	11,5	11,0	0,39	0,36	0,38
	2	6,0	6,0	10,0	10,0	0,6	0,57	0,59
2 Months	1	4,0	3,5	9,5	9,5	0,42	0,39	0,41
	2	6,5	6,0	10,5	11,0	0,62	0,55	0,58

Storage of up to two months at -20°C has little effect on the capacity of the supernatants to inhibit migration.

15. Variation in the Stimulatory Concentrations of PHA, PWM and Con A.

Cultures of the lymphocytes were set up, after stimulation with varying amounts of the above lectins. The cells were pulse stimulated for 2 hours at 37°C in 5% CO_c, and washed twice with PBS and once with MEM. Culture was carried out as detailed in the methods section. Incubation was for three days and the supernatants tested for LIF activity.

The results for PHA are shown in Table 15A.
The results for PWM are shown in Table 15B,
The results for Con A are shown in Table 15C.

Note that the experiment with PHA was done in quadruplicate, whereas the experiment with PWM and Con A was done in duplicate. All had two test wells and one control well per plate.

TABLE 15A

Conc. PHA/3ml of Culture Media		Radius of Migratory Area			M.I.		Mean
		Test		Control			
		a	b		a	b	
5ug	1	9,0	9,0	9,0	1,0	1,0	
	2	8,5	9,0	8,5	1,0	1,0	
	3	8,5	9,5	9,0	0,94	0,95	
	4	8,5	8,5	8,5	1,0	1,0	0,99
10ug	1	5,0	5,0	10,0	0,50	0,50	
	2	4,5	4,5	8,0	0,56	0,56	
	3	4,5	4,5	8,0	0,56	0,56	
	4	6,0	6,0	9,5	0,63	0,63	0,56
15ug	1	3,0	3,5	7,0	0,43	0,5	
	2	4,0	4,0	9,0	0,44	0,44	
	3	3,0	3,0	7,0	0,43	0,43	
	4	3,5	3,5	8,0	0,44	0,44	0,44
20ug	1	4,0	4,0	6,5	0,62	0,62	
	2	8,5	9,0	11,0	0,77	0,82	
	3	8,5	8,5	11,0	0,77	0,77	
	4	9,0	9,0	11,0	0,82	0,82	0,75
25ug	1	9,0	9,5	10,0	0,9	0,95	
	2	8,0	8,0	9,5	0,84	0,84	
	3	8,5	9,0	10,0	0,81	0,86	
	4	9,0	9,0	10,0	0,9	0,9	0,88

At least 10 to 15ug of PHA is needed for pulsing the lymphocytes in order to produce LIF. More than this causes a decrease in inhibition of migration.

TABLE 15B

Conc. PWM/3ml of Culture Media		Radius of Migratory Area			M.I.		Mean
		Test		Control			
		a	b		a	b	
6ug	1	8,0	8,0	11,0	0,73	0,73	
	2	6,5	6,5	9,0	0,72	0,72	0,73
12ug	1	3,5	3,5	8,0	0,44	0,44	
	2	4,5	4,5	10,0	0,45	0,45	0,45
18ug	1	2,5	2,5	8,0	0,31	0,31	
	2	3,0	3,0	10,0	0,3	0,3	0,31
24ug	1	3,0	3,0	9,0	0,33	0,33	
	2	3,0	3,0	9,0	0,33	0,33	0,33
30ug	1	2,5	3,0	9,0	0,28	0,33	
	2	2,5	2,5	8,0	0,31	0,31	0,31

18ug of PWM per 3ml culture media is needed to produce LIF which gives a maximum inhibition of migration of PMN's. Below this figure, less inhibition of migration occurs and above this figure, there is no significant difference in the LIF produced.

TABLE 15C

Conc. Con A/3ml of Culture Media		Radius of Migratory Area			M.I.		Mean
		Test		Control			
		a	b		a	b	
50ug	1	6,0	6,5	8,0	0,77	0,81	
	2	6,5	6,5	8,5	0,77	0,77	0,78
100ug	1	3,0	3,0	8,0	0,38	0,38	
	2	3,0	3,0	8,0	0,38	0,38	0,38
150ug	1	4,0	4,0	9,5	0,42	0,44	
	2	3,5	4,0	9,0	0,39	0,44	0,42
200ug	1	8,0	8,0	9,0	0,88	0,88	
	2	8,0	8,0	9,0	0,88	0,88	0,88
300ug	1	8,5	8,0	9,5	0,90	0,84	
	2	8,5	8,5	9,0	0,94	0,94	

At least 100 to 150ug of Con A is needed per 3ml culture media to produce maximum LIF activity. Above or below this figure, has a detrimental effect on LIF production.

16. Variation in Culture Time After Pulsing with PHA, PWM and Con A.

The lymphocytes were set up after pulse stimulation with either 5ug PHA/ml, 20ug PWM/ml or 100ug Con A/ml.

All other parameters were as normal except that cultures were of 7ml volume with 1 X 10^6 cells/ml and in duplicate. They are labelled a and b in the table. 1ml was removed from each culture flask on each day for seven days and assessed for LIF activity.

The results are shown in Table 16A for PHA, 16B for PWM and 16C for Con A.

TABLE 16A (PHA)

Incubation Time	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
1 Day	4,0	3,5	13,0	11,0	0,31	0,32	
	3,5	2,5	9,5	10,0	0,37	0,25	0,31
2 Days	4,0	4,5	11,0	11,0	0,36	0,41	
	4,5	3,5	11,5	10,5	0,39	0,33	0,37
3 Days	4,0	4,5	11,0	11,0	0,36	0,41	
	4,5	4,5	11,0	11,0	0,41	0,41	0,40
4 Days	6,0	6,5	11,0	12,0	0,55	0,54	
	6,0	6,0	12,0	12,0	0,5	0,5	0,52
5 Days	7,0	8,0	11,0	12,0	0,64	0,67	
	7,5	8,0	11,0	11,5	0,68	0,7	0,67
6 Days	9,0	9,0	12,0	12,0	0,75	0,75	
	8,5	8,5	11,0	11,0	0,77	0,77	0,76
7 Days	11,0	8,5	14,0	11,0	0,79	0,77	
	9,0	11,0	11,0	14,0	0,82	0,79	0,79

Pulsing the lymphocytes with PHA, produces maximum LIF activity after 24 hours but there is little change in LIF activity until after 3 days culture, when the activity of the LIF decreases significantly after each twenty four hour period.

TABLE 16B (PWM)

Incubation Time	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
1 Day	7,0	6,5	9,0	9,0	0,78	0,72	
	7,5	7,5	9,5	9,0	0,79	0,83	0,78
2 Days	6,0	6,0	9,0	9,0	0,67	0,67	
	6,5	6,0	9,0	9,5	0,72	0,63	0,67
3 Days	3,5	3,5	10,0	10,0	0,35	0,33	
	3,5	4,0	10,0	10,0	0,35	0,4	0,36
4 Days	3,5	4,0	10,0	10,0	0,35	0,4	
	4,0	4,0	10,0	10,0	0,4	0,4	0,39
5 Days	4,5	5,0	9,0	9,5	0,5	0,53	
	5,0	4,5	9,0	8,5	0,56	0,53	0,53
6 Days	7,0	7,0	10,0	10,0	0,7	0,7	
	7,5	7,5	9,5	9,5	0,79	0,79	
7 Days	8,0	7,5	9,5	9,0	0,84	0,83	
	7,5	7,5	9,5	9,5	0,79	0,79	0,81

Pulsing with PWM produces maximum LIF activity after 3 to 4 days culture. After this time, LIF activity decreases markedly.

TABLE 16C(Con A)

Incubation Time	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
1 Day	5,0	5,5	10,0	9,5	15,0	0,58	
	5,0	5,0	10,0	9,5	0,5	0,53	0,53
2 Days	4,0	4,0	10,0	10,0	0,4	0,4	
	4,0	4,0	10,0	10,0	0,4	0,4	0,4
3 Days	3,5	3,5	9,5	9,0	0,37	0,39	
	3,5	3,5	9,0	9,5	0,39	0,37	0,38
4 Days	5,0	5,5	9,0	9,5	0,56	0,58	
	4,5	4,5	9,0	9,0	0,5	0,5	0,54
5 Days	5,0	5,0	9,0	9,5	0,56	0,53	
	5,5	5,0	9,0	9,0	0,61	0,56	0,57
6 Days	7,0	7,5	9,0	9,5	0,78	0,79	
	7,0	7,0	9,0	9,0	0,78	0,78	0,78
7 Days	8,5	8,5	9,0	9,0	0,94	0,94	
	8,5	8,5	9,0	9,0	0,94	0,94	0,94

Pulsing with Con A produces maximum LIF activity after 3 days and after this time, LIF activity decreases markedly.

17. Variations in Pulsing Time.

This experiment was only carried out, using PHA. The lymphocytes were pulse stimulated with 5ug PHA/ml for varying times before being washed by the usual procedure. The cells were incubated for 60 hours and the supernatants tested as usual. This was done in duplicate, the two cultures being labelled a and b.

The results are shown in Table 17.

TABLE 17

Pulse Time	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
½ Hour	8,0	8,0	9,5	9,0	0,85	0,89	
	9,0	8,5	9,5	9,0	0,95	0,94	0,91
1 Hour	6,5	6,5	9,0	9,0	0,72	0,72	
	7,5	7,0	9,5	9,0	0,79	0,78	0,75
1½ Hours	4,0	4,5	9,0	9,5	0,44	0,47	
	4,5	4,5	10,0	9,0	0,45	0,5	0,47
2 Hours	2,5	2,5	10,0	9,5	0,25	0,26	
	3,0	3,0	10,0	10,0	0,3	0,3	0,28
3 Hours	3,0	3,5	10,0	10,0	0,3	0,35	
	3,5	3,5	9,5	9,5	0,37	0,37	0,35
4 Hours	3,5	4,0	11,5	11,0	0,3	0,36	
	3,0	2,5	10,0	8,0	0,3	0,31	0,32

A pulse time of at least 2 hours with PHA is needed to produce maximum LIF activity. An increase in the pulse time has no effect on the LIF production, in fact it may decrease slightly, but this is insignificant, even after 4 hours.

SECTION 3

LIF Produced From The Mixed Lymphocyte Reaction (MLR).

18. Variation in the Quantity of Stimulated Cells.

Stimulatory cells were incubated with 50ug mitomycin per 1 X 10^5 cells in 1ml for 30 minutes, washed two times with PBS and once with MEM to remove the mitomycin. The stimulatory cells were suspended in varying amounts of MEM to give varying quantities of stimulatory cells per ml. 1ml of stimulatory cells was added to 1 X 10^6 responder cells per ml and the mixture cultured as previously described for 3 days and the supernatant tested for LIF activity.
The experiment was done in duplicate.
The results are shown in Table 18.

TABLE 18

No. of Stimulatory Cells	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
1×10^4	4,0	4,0	10,0	10,0	0,4	0,4	
	3,5	4,0	10,0	9,5	0,35	0,42	0,39
5×10^4	2,5	2,5	9,5	9,0	0,27	0,28	
	3,0	2,5	10,0	10,0	0,3	0,25	0,28
1×10^5	2,0	2,0	10,0	9,5	0,2	0,21	
	2,0	2,0	9,5	9,5	0,21	0,21	0,21
$2,5 \times 10^5$	2,0	2,0	10,0	9,5	0,2	0,21	
	2,0	2,0	10,0	10,5	0,2	0,19	0,20
5×10^5	1,5	1,5	10,0	10,0	0,15	0,15	
	2,0	1,5	10,0	10,0	0,2	0,15	0,16
$7,5 \times 10^5$	1,0	1,0	10,0	10,0	0,1	0,1	
	1,0	1,0	10,0	9,5	0,1	0,11	0,10
1×10^6	1,0	1,0	9,5	9,5	0,1	0,11	
	1,0	1,0	10,0	10,0	0,1	0,1	0,10
3×10^6	1,0	1,0	9,5	9,5	0,11	0,11	0,11

In the mixed lymphocyte reaction, LIF production is increased by the addition of more stimulatory cells. At least $7,5 \times 10^5$ stimulatory cells are needed to produce maximum LIF activity as shown by the M.I. measured by inhibition of migration. Increasing the number of stimulatory cells does not affect LIF activity.

19 Variation in Culture Time in the MLR.

This experiment is almost identical to Experiment 16, except that the responder cells were stimulated, as in the previous experiment, using 1×10^5 stimulator cells for 1 to 6 days.

The experiment was done in duplicate.

The results are shown in Table 19.

TABLE 19

Time	Radius of Migratory Area				M.I.		Mean
	Test		Control				
	a	b	a	b	a	b	
1 Day	7,5	8,0	8,0	8,5	0,93	0,94	
	10,0	10,0	10,5	11,0	0,95	0,91	0,93
2 Days	5,0	5,5	8,5	8,5	0,59	0,65	
	5,5	5,5	9,0	9,0	0,61	0,61	0,62
3 Days	3,0	3,5	9,0	9,5	0,33	0,37	
	3,5	3,5	10,0	10,0	0,35	0,35	0,35
4 Days	2,5	2,5	9,5	10,0	0,26	0,25	
	2,5	2,5	9,5	9,5	0,26	0,26	0,26
5 Days	4,0	4,5	10,0	10,5	0,40	0,43	
	4,0	4,0	9,5	9,5	0,42	0,42	0,42
6 Days	6,5	6,0	11,0	11,0	0,59	0,55	
	7,0	7,0	10,5	10,0	0,67	0,7	0,63

In the MLR, four days incubation time produces maximum LIF activity. LIF activity decreases after this time.

20. Effect of E. coli Lipopolysaccharide on PMN Migration.

Instead of using a lectin to stimulate the lymphocytes, a phenol extraction of the lipopolysaccharides from E. coli serotype 0128 : B12 was used.

Concentrations of LPS, using from 10ug up to 1mg per 3ml of culture media, containing 1 X 10^6 lymphocytes per ml.

This experiment was done in duplicate.

The results are shown in Table 20.

TABLE 20

Conc. of LPS	Radius of Migratory Area				M.I.
	Test		Control		
	a	b	a	b	
10ug	8,0	8,0	8,0	8,0	-
	10,5	10,5	10,5	10,5	-
20ug	8,0	8,0	8,0	8,0	-
	10,0	10,0	10,0	10,0	-
40ug	8,0	8,0	8,5	8,0	-
	10,5	10,0	10,0	10,0	-
60ug	8,0	8,0	8,0	8,0	-
	10,0	10,0	10,0	10,0	-
80ug	8,5	8,5	8,5	8,5	-
	10,5	10,0	10,0	10,0	-
100ug	8,0	8,0	8,5	8,0	-
	10,0	10,5	10,0	10,0	-
250ug	8,0	8,0	8,0	8,0	-
	10,0	10,5	10,0	10,0	-
500ug	8,0	8,5	8,0	8,0	-
	10,5	10,0	10,0	10,0	-
750ug	8,5	8,5	8,0	8,0	-
	10,0	10,0	10,0	10,0	-
1000ug	8,0	8,0	8,0	8,0	-
	10,0	10,0	10,5	10,0	-

These results show that there was nothing present in the supernatant which affected the migration of the PMN cells.

DISCUSSION

Macrophage and PMN cell migration from capillary tubes based on the methods of Soberg & Bendixen and Rosenberg & David is widely used in immunological laboratories (Waithe & Hirschorn, 1978 ; Cohen, 1980).

The capillary tube method is tricky and requires two assistants with experience to set up and the sources of variation, such as pH and cell concentration, within this assay, are poorly defined (Morley et al., 1978). The agarose plate method developed by Clausen in 1971, is a very sensitive method for measuring the effect of lymphokines on either the migration of leucocytes or macrophage cells (Morley et al., 1978). The assay method is relatively simple compared to the capillary tube method (Weisbart & Mickey, 1977 ; Willoughby et al., 1978), and it may be that this method will become the routine method of the future as it is widely used now (Schaak et al., 1978). Before any method becomes routine, it is necessary to know the factors influencing the results and more important, the reliability of the method. The experimental section of this thesis has gone some way towards achieving this goal.

The first group of experiments, was an attempt to standardise the methodology and to obtain the optimum conditions for the assay. As the basis for this procedure is the agarose plate, the various parameters of the formulation of the agarose plate were examined,

as there are a number of agarose types available commercially, the price of which is very variable. Several of these agaroses have been used in the agarose plate method eg Lavergne & Harrington (1978) used Sea Plaque Agarose from Marine Colloids ; Borowsky & Lawrence (1979) used Agarose from Biorad. Because of these reasons, the type of agarose used and its effect on migration was examined.

Experiment 1. shows there can be considerable variation in the migration of the cells using the six types of agarose selected for this experiment under standard conditions. It could be that the poor migration in Type C agarose could be improved by changing other parameters, but for the purpose of these experiments, only one parameter was altered at a time. The results show that migration was best, using Sigma Type 2 agarose which coincidentally, was one of the cheapest used. Consequently this agarose was used in all other experiments.

In Experiment 2., the effect of employing different types of sera was investigated. As can be seen from the results, the type of sera used had little effect on the mean radius of the migratory area, except that the use of heat inactivated sera did result in a lowered migration. Foetal calf serum was perhaps slightly better than horse serum, but as horse serum is cheaper, it was the serum of choice and was employed for the rest

of the experimental work. The advantage of using Miles Laboratories horse serum was that it could be stored at 4°C. Unfortunately this product is no longer available.

It may be that heat inactivated sera contains more denatured substances which can slightly inhibit migration. Human AB serum should be avoided, since there could be an immunological reaction between the antibodies in the serum and the lymphocytes (McIntyre & Cole, 1969).

The same batch of horse serum was used for all the experiments, so the only variation between experiments, as far as the serum was concerned, was one of age.

pH has an important effect on the migration of leucocytes and it can be seen from Fig. 2, that the optimum pH of the migration is 7,4. This is in agreement with Willoughby et al., (1978) who assessed the variation of the pH of the plates on the actual response of the PMN leucocytes to LIF. The results show that migration is more sensitive to acid conditions than to alkaline conditions. David et al., (1964) reported that lowering the pH of the gel, inhibited the action of LIF on guinea pig macrophage cells but do not give details of the pH changes. Hughes, (1973) found that pH changes from 7,2 to 8,0 did not affect

guinea pig LIF activity, which is in accordance with the results shown here, but the effect of lower pH levels was not studied by him. The cells themselves may be affected by low values which causes the decrease in migration.

Further confirmation of the effect of pH on migration is seen in Table 5, where it can be seen that if the plates are incubated in a carbon dioxide free atmosphere, there is no migration as a result of metabolism producing a lowered pH. The presence of carbon dioxide will counteract this. The lowering of the pH in these circumstances is evident from the change in colour of the phenol red in the agarose medium.

The use of HEPES as a buffer in the preparation of the agarose plates was not conducive to a good migration, migration being almost inhibited completely. It should be noted here that the HEPES present in the supernatant was diluted to at least below 1mM and this concentration does not affect migration.

The optimum temperature for migration was 37°C, rather than cooler conditions, as can be seen from Table 5. As the migratory process is a vital process, it follows that the effect of temperature on migration will be similar to its effect on most other biological processes.

Time is a very important consideration in the usefulness of any routine or even research technique and Table 4. shows the effect of time on the migration and obviously the shorter the time to produce meaningful results, the better it is. Very little migration takes place for the first two hours, as presumably during this time, the cells are recovering from the procedures used in their preparation, as the cells have had some traumatic treatment, particularly during the lysis of the erythrocytes (Glasser & Fiederlein, 1979).

The migratory area is too small for measurement, in the early stages of incubation time, and as the migration, when LIF is used, would be even less, the resulting M.I. would not be very accurate. When one considers that the radial measurement cannot be 100% accurate and a 0,2cm error is much higher at a 1cm migration than the same error at a 10cm migration.

There were no timed incubations between five and fifteen hours as the plates and cells take at least three hours to prepare. This would have meant measuring the migration during the night which was not convenient and as it was deemed necessary only to obtain the optimal time for migration, a ten hour interval was used after the five hour incubation time. (Presumably the migration per unit time would be linear).

Incubation for fifteen hours produces a good migration which will continue to enlarge up to twenty four hours which was the maximum duration used in this experiment, but as is stated already, the area becomes more difficult to measure, owing to scattering of the cells at the periphery. As one can see from the results in Table 4., the rate of migration does slow down after fifteen hours. This time of 15 hours does not agree with Glasser & Fiederlein, (1979) who state 4 - 5 hours as being the optimum time but compare favourably with Morris et al., (1976) who used 18 hours and Bullen & Losowsky, (1977) who used 20 hours. Glasser & Fiederlein, (1979) suggest that autolysis of the PMN's occurs as cell debris is present between the intact PMN's after migration has taken place for longer than five hours. This does happen but it does not appear to affect the migration. Thus, the agarose plate method of measuring cell migration is superior, as far as speed is concerned, when compared to the microcapillary methods which take 24 hours (Hahn et al., 1976 ; Lavergne & Harrington, 1978).

As has been stated in the methodology section, it is impractical to remove all the red cells without harming some of the PMN's and Experiment 6 shows the effect of the presence of erythrocytes on the migration. The radii of the migratory areas in this experiment

are variable due to the fact that the figures do vary somewhat from day to day. (This will be discussed later). It can be concluded, that up to 80% contamination with erythrocytes has no appreciable effect on the migration and unless the contamination is above this figure, a second haemolytic step is not necessary. (Willoughby et al., (1978) used a second flash haemolysis with distilled water to remove the red cells and Weisbart & Mickey, (1979) used dextran sedimentation to achieve the same effect, but it is evident from the results, that these steps are not necessary.

The number of cells per well used, varies, depending upon the individual researcher. Eg Weisbart & Mickey, (1977) used 2 X 10^5 cells in 4ul and Orr & Ward, (1978) used 2,5 X 10^5 cells in 10ul. From Experiment 7., the optimum number of cells to add to the wells is 2 X 10^5 per either 5ul or 10ul M199, which suggests that the volume added to the wells is not as important as the absolute number of cells used. Thus both the above researchers used the optimum number, but only varied the volume. The number of cells used can be seen to be fairly critical as a decrease or increase by 50%, almost halves the radius of the migratory area.

The literature on leucocyte migration does not help

one to ascertain whether the leucocytes used in the migration procedure are from one donor or several eg. (Glasser & Fiederlein, 1979 ; Urist et al., 1976 ; Lavergne & Harrington, 1978 ; Morris et al., 1976 ; Cochrane et al., 1978). Experiment 8., shows the results of the migration of the cells from three sets of four donors. It is clearly seen that mixing the cells from at least three donors definitely improves the migration and that out of the twelve donors, two produced a poor migratory response which is negated by the mixing. Therefore, it seems essential that the leucocytes should be obtained from different donors and mixed before use.

As it takes some time to make up the agarose plates, it would be convenient if the plates could be made up in batches ; the results of Experiment 9., show that the plates can be stored for at least one day at either $4^{o}C$ or at room temperature without having any significant effect on the migration. Storage at $37^{o}F$ was of no use at all and storage for longer than two days, at room temperature or $4^{o}C$, caused a significant deterioration in the plates as far as migration was concerned. The deterioration at the higher temperatures is not surprising as the preparation of the plates was not under strictly sterile conditions and microbial growth may take place to interfere with migration.

This could happen at $4^{o}C$ after a longer time, although there was no visible evidence of this.

As one of the purposes of this thesis is to look at lymphokine production from lymphocytes stimulated with lectins, Experiment 10., shows the effect of the lectins alone, on the migration of the PMN cells using various concentrations of each. Pulsing the cells with the lectins was also looked at, in order to see whether this had any effect on the migration. The results show that the lectins are not strongly bound to the PMN cells, as no effect on migration was observed after a 2 hour pulse. If lectins are in the cell suspension, they cause an inhibitory effect on migration. With Con A at 50ug/ml and above, no migration takes place and it is possible that the Con A is toxic to the cells. The concentration of Con A that produces a good inhibition is between 12,5ug/ml and 25ug/ml. PWM is effective at concentrations of 1ug/ml, in inhibiting migration, whereas PHA, at a concentration of 30ug/ml is not as effective as 1ug/ml of PWM.

Since PWM causes a strong inhibition of migration at low concentrations, it is important when using PWM as a stimulant, to ensure that pulsing of the lymphocytes is carried out and the pulsed cells are washed thoroughly to remove the PWM as completely as possible

from the supernatant. It is significant that the results from Table 11c, show that pulsing the PMN's with PHA has some effect on the migration, especially at higher concentrations. Therefore, it seems as if PHA may have some effect on the membranes of the PMN cells, causing an inhibition of migration or is taken up by the cells at high concentrations where it can continue to have its effect on the migration of the PMN's even after washing. Willoughby et al., (1978) reported that Con A at a concentration of 8ug/ml does not affect migration and this is confirmed here.

In Section 2, the effects of LIF on migration were looked at. In Tables 12a and 12b, a comparison is made between LIF obtained from stimulated lymphocytes from black and white donors and as can be seen, the LIF production by blacks and whites is similar, giving M.I.'s of 0,54 $\pm$ 0,24 for whites and 0,54 $\pm$ 0,15 for blacks.

The migratory indices, as shown above, from normal donors, compare favourably with previously published results. Willoughby et al., (1978) report an M.I. of 0,49 $\pm$ 0,2. Glasser & Fiederlein, (1979) report an M.I. of 0,59 $\pm$ 0,7. As LIF is a glycoprotein (Kasakura, 1970 ; Lawrence & Landy, 1969), with enzymic activity (Bendtzen, 1981), its stability is an important aspect of its study. Experiment 14., shows the effect of

storage at -20°C and thawing, on LIF activity.

As would be expected, freezing and thawing does affect LIF activity, especially after three thawing processes, when LIF activity drops to near zero. Proteins and glycoproteins are denatured by freezing and thawing repeatedly and one would expect the LIF activity to decrease gradually but this experiment confirms Experiment 13, in that the action of LIF is an all or none response as the LIF activity ceases abruptly after 3 thawings, but shows no change in activity after 2 thawings. If many tests are to be carried out on one supernatant from lymphocyte culture, they should be stored as aliquots in order to avoid freezing and thawing. Table 14b shows that LIF activity is not affected by storage of up to 2 months at -20°C. Bendtzen, (1980) states that LIF has limited stabiltiy in frozen supernatants but does not state any time.

In Experiment 15., the effect of varying the concentrations of the three lectins used in LIF production are looked. In considering these results, it is important to ascertain just how reliable the migratory index is. Erad, (1974) considered migratory indices of 0,9 and less as being significant for the presence of LIF, whereas Bloom & Bennet, (1971) and Salvin et al., (1974) took migratory indices of 0,8 and lower as being significant. As many of the migratory indices obtained in the course

of this work are between 0,8 and 0,9 and this was obtained using several controls and test samples, these migratory indices have been taken to show an inhibition of migration, and those above 0,9 have been taken to show no significant inhibition of migration. Table 15a shows the effect on the production of LIF by PHA which acts on T-lymphocytes. 5ug/3ml of lymphocyte suspension has no effect, whereas 10ug/3ml and 15ug/3ml, under the conditions of the experiment, produces a maximum response. At 20ug/3ml and 25ug/3ml, the migration of the lymphocytes is only slightly inhibited. It has already been stated that it appears that LIF produces an all or none response but it is probable that very small quantities of LIF will produce minimal inhibition of migration and it appears that using a high stimulatory dose of PHA to pulse the lymphocytes produces only very small amounts of LIF. This could be due to the fact that the cells are actually inhibited from producing LIF but not killed at this concentration as the trypan blue exclusion test showed 85 - 90% live cells present at this concentration.

Table 15b, shows the results of the PWM stimulation. The 6ug and 12ug stimulatory doses seem to produce very small amounts of LIF which do not produce maximum inhibition. At concentrations of 18ug/3ml and above of PWM, the M.I. remains constant.

The migratory index is smaller than that produced by PHA stimulation, but as PWM stimulates both T- and B-cells and each may produce an LIF, the low migratory index is probably due to a combined effect of both LIF's. PWM does not cause inhibition of LIF production even at 30ug concentrations.

Con A stimulation (Table 15c) is not active at concentrations of below 50ug. 50ug produces a subminimal amount of LIF, 100 and 150ug, an optimum amount and at 200 and 300ug, an insignificant amount. As Con A itself, does not affect appreciably the migration of PMN's, the higher concentrations must inhibit LIF production as does probably the PHA. As Con A can stimulate B-cells slightly as well, it may be that one of the cell populations is not affected as much, by the high concentrations of Con A. The low migratory index produced by the supernatants from Con A stimulated cells may be, as for PWM, the result of a combined effect of two LIF's, one from each cell population.

In Experiment 16., the time of incubation with the lectins was examined. With PHA, stimulation for one to three days, produced enough LIF to cause a maximal inhibition of migration and after day three, the inhibitory effect of the LIF decreases, at first rapidly and then more gradually. Whereas with PWM, it does not produce maximal inhibitory amounts of LIF

until day three and Con A, day two. All lectins produced a decrease in LIF production after three to four days. This could be due to the death of the lymphocytes which does occur rapidly after three days. Usually cell survival rates were approx. 85 - 90%, after three days but this would decrease to 10 - 15% after five days. This of course, is expected as the nutrients are used up and the condition of the culture medium changes due to metabolite production by the cells themselves. This cell death would stop production of LIF and at the temperature of 37°C, glycoproteins in solution may denature and lose some of their biological activity, or it could be an enzymic degradation (Bendtzen, 1976). The variation in decrease of LIF activity using the different lectins is slight and any differences are probably due to the denaturisation of the LIF which is not a uniform event and it may be that in the 'semi-denatured' state, some biological activity may remain.

It is obvious from this experiment, that PHA is the most efficient activator of lymphocytes, producing enough LIF after only one day's culture. This is in agreement with Kolb et al., (1971) ; Shacks & Grainger, (1971) and Lewis et al., (1976).

In Experiment 17., the pulsing time using PHA as a stimulant was studied. Maximal inhibitory amounts of LIF are produced after a two hour pulse which is in

agreement with the previous results that once maximal inhibitory amounts of LIF have been produced, more LIF will not produce any increase in migration inhibition. The exact mechanisms by which LIF inhibits the migration of PMN's is as yet unknown, but it may be due to increased adhesiveness of the PMN's which may be due to a decrease of c-AMP in the cells (Lomnitzer et al., 1976a ; Lomnitzer et al., 1976b). Bendtzen (1980) proposes that LIF is an allosteric enzyme regulated by c-GMP. The action of the LIF is therefore probably at the cell membrane level, there being receptors on the PMN's (Morley et al., 1978) and once these receptors are saturated with the LIF ie at maximum inhibitory level, using excesses of LIF will have no effect.

Experiment 18., looks at the mixed lymphocyte reaction, using varying numbers of mitomycin C treated lymphocytes as stimulator cells on the responder lymphocytes. Even using comparatively small numbers of stimulatory cells, the culture supernatant contains enough inhibitory substances to produce a considerable effect on the migration of PMN cells. Very high concentrations of stimulatory cells, that is above 1×10^6 cells to 1×10^6 responder cells produce enough of inhibitory substances to produce a mean migratory index of 0,1 which is the lowest observed in all the experiments. This seems to indicate that more than one inhibitory

substance of migration is produced which may or may not contain LIF. It would be interesting here to try and evaluate the type or types of inhibitory factors and also to carry out this type of experiment after having HLA typed the responder and stimulator cells, to see the effect of differing tissue types of the stimulator cells on the responder cells.

In Experiment 19., the production of inhibitory factors was followed with time and unlike the reactions with mitogens, there is a gradual increase of inhibitory substances up to day four (longer than that for mitogens). This is in agreement with the results of Rasanen et al., (1978). Then there was a gradual decrease, probably due to the same reasons as were surmised previously ie denaturation of the inhibitory substances. The production of inhibitory substances during the MLR requires further study.

Experiment 20, shows the lack of effect of lipopolysaccharides from E. coli on human lymphocytes as far as LIF production is concerned. These substances are selectively mitogenic on B-lymphocytes from mice (Greaves & Janossy, 1972). The lack of results on human lymphocytes is in agreement with that of Gery et al., (1972), in that no LIF is produced.

In conclusion, there seem to be differences in the

effect of mitogenic stimulation on both T- and B-lymphocytes in that more than one inhibitory substance is produced. Many other substances are known which are mitogenic and further work is warranted, especially in the isolation of the LIF fraction in order to carry out quantitative experiments on the factors involved in the inhibition of PMN migration.

PART 2

INTRODUCTION

Macrophage cells play an important part in both the afferent and efferent phases of the immune response. In the afferent phase, they partake in the uptake, concentration and transfer of the antigen to immunocompetent cells (Nelson, 1969 ; Fishman, 1959 ; Bona et al., 1972). In the efferent phase of cell mediated immune reactions such as, tumour destructions (Lohman-Mathes et al., 1973), macrophage involvement is brought about by soluble factors of the nature of 1. cytophilic antibodies (Boyden & Sorkin, 1960) 2. 'arming factors', produced by T-cells and apparently different from immunoglobulins (Lohman-Mathes et al., 1973 ; Evans et al., 1973) and perhaps the migration inhibition factor (Bloom & Bennet, 1966).

One of the so called 'arming factors' is MAF as mentioned in the general introduction and this factor which has several effects on macrophage cells (Barnet et al., 1968 ; Godal et al., 1971 ; Nathan et al., 1971) has as two of its effects, the ability to cause macrophage cells to adhere to and spread on surfaces, such as glass and plastic in vitro and tissues in vivo, particularly tumour cells (Piessens, 1978). It may be that both these actions are related and if so, a method for measuring the adherence in vitro would be a useful method for measuring MAF activity.

This part of this thesis is mainly concerned with the development of such a method.

Macrophage cells can be obtained from humans by peritoneal dialysis or by peritoneal washing of recently dead cadavers (Stuart et al., 1978). Their isolation from peripheral blood seems to be an unprofitable procedure (Stuart et al., 1978). They can also be obtained from blister fluid, from blisters which can be produced by vesicants, such as cantharadin (Chang & Yad, 1979). These methods are not suitable for routine purposes for obvious reasons.

Macrophage cells from small mammals can be obtained by peritoneal lavage. Unfortunately the yield of cells is low except in the case of mice and rats but can be increased by the injection of various substances such as liquid paraffin, glycogen, thioglycollate broth etc prior to aspiration of the cells (Stuart et al., 1978).

Macrophage and/or leucocyte adherence inhibition has been measured by at least three methods 1. a capillary tube method which is rather complicated. Urist et al., (1976), used this method to study antigens produced by patients with cancer which specifically cause adherence of autologous macrophage cells, 2. Holan et al., (1974) used a test-tube method again for studying tumour antigens and their effect on macrophage adherence and 3. Halliday & Miller, (1972), who counted the cells adhering to glass or plastic surfaces

such as cover slips. The third method is a laborious procedure if many tests are to be done on substances which can cause macrophage adherence.

The experimental details which follow are in fact a combination of the first two methods but are specifically tailored to the measurement of MAF.

The method to be described, makes use of an electronic cell counter to detect changes in the number of cells in the supernatant of a macrophage suspension after incubation in plastic tubes. As this is a new method, the experimental work is mainly concerned with the development of this method in order to determine whether it will or will not be a suitable tool for measuring MAF activity of supernatants from lymphocyte culture.

SURVEY OF METHODOLOGY

1. Preparation of Macrophage Cells.

As previously stated, mice and rats are the only small animals which are convenient to use in the preparation of macrophage cells. Other animals do not have sufficient of these cells present under normal conditions for most experimental purposes (Stuart et al., 1978), especially that of adherence, as fairly large numbers are needed for this. The yield of macrophage cells from these animals can be increased by injection into the peritoneal cavity of proteose peptone, (Wood et al., 1979), glycogen (Kumagai et al., 1979), purified protein derivative of Mycobacterium tuberculosis (Cousin & Patty, 1975) glycerol tricaprate (Stuart et al., 1978).and many other compounds for several days (Stuart et al., 1978). These substances do increase the yield of macrophage cells greatly but have certain disadvantages which are:

1. The chemical cyto-profile is decreased.
2. Macrophages isolated may contain phagocytosed granules of the inducing agent.
3. PMN's and lymphocytes are increased in the exudate.
4. Clumping of the macrophage cells occurs (Stuart et al., 1978).

In the experiment described, the cells were obtained from the peritoneal cavity of animals without having used any of the above stimulating substances, because of the above reasons which may or may not affect the MAF activity in the cells.

2. Macrophage Adherence.

Halliday & Miller (1972), using cover slips, showed that macrophage cells capable of adherence to glass are inhibited by the addition of an antigen to which the animal has been presensitized. The adherent cells were counted by microscopy, both before and after addition of antigen. Using this method, one can use MAF instead of the antigen to obtain the effect of the MAF on adherence. Holan et al., (1974), counted the cells in the MAF containing supernatant, using a Burker chamber in a similar experiment. Urist et al., (1976), used a Coulter counter to count the cells in the supernatant. They used leucocytes and the equation given below to express the percentage leucocyte adherence (PLA).

$$PLA = \frac{\text{Mean Number of Non-Adherent Cells Without Incubation.} - \text{Mean Number of Non-Adherent Cells After Incubation.}}{\text{Mean Number of Non-Adherent Cells Without Incubation.}} \times 100$$

This equation can be modified to express the adherence of macrophage cells to surfaces as will be discussed in the experimental section.

Adherence of macrophage cells to surfaces is dependant upon the presence of calcium and/or magnesium ions (Trotter & Quintana, 1981) and can be prevented from adherence by the addition of a chelating agent such as, ethylenediamine tetraacetic acid (EDTA) (Rubinowitz, 1964 ; Bloom & Bennet, 1966). Other agents which have been used to prevent adherence are trypsin (Churchill et al., 1975) and lignocaine (Rabinovitch & De Stefano, 1975). Trypsin, being a protease, in all probability affects MAF, so it is not suitable for studies involving MAF. Lignocaine alters the size of the macrophage cells by causing swelling and vacuolation (Wood et al., 1979), and will affect the counting procedure, so is not a useful method when automatic counting methods are used. The coating of surfaces with heat inactivated foetal calf serum (Kumagai et al., 1979), increases the binding of macrophage cells to the surface. This is a useful procedure to employ in the study of macrophage adherence.

METHODS

1. Preparation of Macrophage Cells.

Mice were anaesthetised by an intramuscular injection of pentobarbitone and the abdominal area cleaned with hibitane solution. 10ml of medium 199, containing 5 units of heparin per ml was injected into the peritoneal cavity along the mid-anterior line, taking care to avoid puncture of the gut. The abdomen was prodded to circulate the injected fluid. After two to three minutes, the fluid was aspirated using a 15 gauge needle into a sterile test-tube. Usually about 8ml of fluid could be aspirated. Cells aspirated from several mice were combined and centrifuged at 200 g for five minutes and the macrophage pellet washed twice with M199. The cells were examined after staining with Turks stain and were always between 85 - 90% macrophage cells. The final concentration of cells was adjusted if necessary to give a suspension of 1×10^6 cells per ml. The suspension was supplemented with 10% heat inactivated foetal calf serum.

2. Preparation of Tubes for Adherence Assay.

5ml sterile capped polystyrene tubes were filled with heat inactivated foetal calf serum and stored at $4^{\circ}C$. Before use the tubes were emptied as completely as possible and 0,9ml of macrophage

suspension added. To the control tube was added 0,1ml PBS and to the others various chelating agents and/or lymphokines as described in the results section. Cells were counted using a Sysmex microcell counter with the white cell discriminator at zero.

MATERIALS

EDTA Na_2	B.D.H.
EDTA Na_3	Riedel De Haen
EDTA Na_4	Sigma
EDTA K_2	Riedel De Haen
Heat Inactivated Foetal Calf Serum	Flow
Pentobarbitone	May & Baker
Capped Polystyrene Tubes 2003 12X75mm	Falcon Plastics

EXPERIMENTAL & RESULTS

1. Adherence of Cells Without the Addition of any Agent.

This experiment was carried out to determine the constancy of macrophage adherence to the walls of the tubes.

In Group A, the cells were from one mouse, while in Group B, mixed cells from two, three or four mice were used. Counts were made after 1 hour, 2 hours and 3 hours. Incubation was at $37^{o}C$ in 5% CO_2.

The figures shown have had a blank count substracted. Before counting, the tubes were inverted carefully to ensure that all non-adherent cells were present in the supernatant.

The results are shown in Table 21A and 21B.
In Table 21B, the results are grouped together to show how the Percentage Macrophage Adherence (PMA), is affected by the number of cells per tube.
(There is no differentiation between whether the cells are from one or several mice in this table).

TABLE 21A

Group A

Counts Without Incubation	Counts After Incubation			PMA %		
cells mm^3	1 Hr	2 Hrs	3 Hrs	1 Hr	2 Hrs	3 Hrs
1100	800	700	600	27,3	36,4	45,5
1100	800	700	700	27,3	36,4	36,4
1200	800	700	600	33,3	41,7	50,05
1500	900	800	700	40,0	46,7	53,3
1400	800	700	700	42,9	50,0	50,0
1300	800	700	700	38,5	46,2	46,2
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	700	700	600	46,2	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1000	800	700	600	20,0	30,0	40,0
1100	800	700	600	27,3	36,4	45,5
1100	700	700	600	36,4	36,4	45,5
1300	700	700	600	46,2	46,2	53,9
1000	700	700	600	30,0	30,0	40,0
1100	800	700	600	27,3	36,4	45,5
1300	800	700	600	38,5	46,2	53,9
1200	800	700	600	33,3	41,7	50,0

TABLE 21A

Group A

Counts Without Incubation	Counts After Incubation			PMA %		
cells mm^3	1 Hr	2 Hrs	3 Hrs	1 Hr	2 Hrs	3 Hrs
1200	800	700	600	33,3	41,7	50,0
1300	800	700	700	38,5	46,2	46,2
			S.D.	6,4	5,6	5,3
			Co.V.	17,9	13,2	10,0
			Mean PMA	35,7	42,4	49,0

TABLE 21A (Continued)

Group B

Counts Without Incubation	Counts After Incubation			PMA %		
cells mm^3	1 Hr	2 Hrs	3 Hrs	1 Hr	2 Hrs	3 Hrs
1000	800	700	600	20,0	30,0	40,0
1100	800	700	600	27,3	36,4	45,5
1100	800	700	600	27,3	36,4	45,5
1100	800	700	600	27,3	36,4	45,5
1000	800	700	600	20,0	30,0	40,0
1000	700	700	600	30,0	30,0	40,0
1200	800	700	700	33,3	41,7	41,7
1300	800	700	700	38,5	46,2	46,2
1300	800	700	600	38,5	46,2	53,9
1100	800	700	600	27,3	36,4	45,5
1100	800	700	600	27,3	36,4	45,5
1200	800	700	600	33,3	41,7	50,0
1300	800	700	700	38,5	46,2	46,2
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
1300	800	700	600	38,5	46,2	53,9
			S.D.	6,6	6,3	5,2
			Co.V.	20,7	15,8	11,0
			Mean PMA	31,9	39,9	47,1

$$\text{Coefficient of Variation (Co.V.)} = \frac{\text{S.D.}}{\text{Mean}} \times 100$$

In Table 21A, in groups A and B, column one shows the number of cells at the start of incubation and is variable.

Columns two, three and four, show the number of non-adherent cells after incubation for 1, 2 and 3 hours respectively. There is a definite decrease in non-adherent cells, the longer the tubes are incubated.

Columns five, six and seven, show the PMA as a percentage after 1, 2 and 3 hours respectively. Incubation for 3 hours gives the lowest coefficient of variation and in all the subsequent experiments, incubation was carried out for three hours.

The number of cells in the supernatant was checked using a haemocytometer and the cells were found to be in the in the same order of magnitude as using the cell counter.

TABLE 21B

Number of Cells Without Incubation	PMA After 3 Hrs.			Mean
1000	40,0	40,0	40,0	
	40,0			40,0
1100	45,5	36,4	45,5	
	45,5	36,4	45,5	
	45,5	45,5	45,5	
	45,5			43,7
1200	50,0	50,0	50,0	
	41,7	50,0		48,3
1300	46,2	53,9	53,9	
	53,9	53,9	53,9	
	53,9	53,9	53,9	
	46,2	46,2	53,9	
	46,2	53,9	53,9	
	53,9	53,9		52,1

This Table groups together the results for Experiment 1., according to the number of cells per tube. The higher the number of cells, the more adherence.

To try and see if there was any reason for this increase in adherence, macrophage cells were added to a glass slide and covered with a cover slip and incubated for three hours. After this time, the cover slip was removed and the slide washed gently with a stream of PBS.

The slide was examined microscopically and it could be seen that the macrophage cells clump together and are not spread evenly over the slide. The more macrophage cells that were present on the slide, the larger the clumps were.

2. Adherence of Cells After the Addition of a Chelating Agent.

Various chelating agents were added to the macrophage cell suspensions.

The following were used in varying concentrations as shown in Table 22.

		Table
Ethylene diamine tetraacetic acid disodium salt	EDTA Na_2	(22A)
Ethylene diamine tetraacetic acid trisodium salt	EDTA Na_3	(22B)
Ethylene diamine tetraacetic acid tetrasodium salt	EDTA Na_4	(22C)
Ethylene diamine tetraacetic acid dipotassium salt	EDTA K_2	(22D)

These agents were made up in PBS and added to the macrophage cells before incubation.

A control was run in duplicate, with each agent and the incubation period was for three hours in each case.

TABLE 22A

Cell Count Without Agent Before Incubation Cells/mm^3	Cell Count Without Agent After Incubation Cells/mm^3	Conc. of EDTA Na_2 uM/ml	Cell Count With Agent After Incubation Cells/mm^3	PMA Without Agent %	PMA With Agent %
1400	600	5	1100	57,1	21,4
1400	600	5	1100	57,1	21,4
1400	600	5	1100	57,1	21,4
1300	600	10	1000	53,9	23,1
1300	600	10	1000	53,9	23,1
1300	600	10	1000	53,9	23,1
1300	600	15	1100	53,9	15,4
1300	600	15	1100	53,9	15,4
1300	700	15	1100	46,2	15,4
1300	600	20	1100	53,9	21,4
1300	700	20	1100	53,9	21,4
1300	700	20	1100	46,2	15,4
1300	700	25	1100	46,2	15,4
1300	600	25	1100	53,9	21,4
1300	600	25	1100	53,9	21,4
1300	600	30	1100	53,9	21,4
1300	600	30	1100	53,9	21,4
1300	600	30	1100	53,9	21,4

The last column gives the PMA as a percentage and shows that disodium EDTA is very efficient in preventing the adherence of macrophage cells ; even at low

concentrations, there is no difference in the prevention of adherence when compared to the higher concentrations of EDTA Na_2. The results show that between sixty and eighty per cent less cells are adherent in the presence of EDTA Na_2.

TABLE 22B

Cell Count Without Agent Before Incubation Cells/mm^3	Cell Count Without Agent After Incubation Cells/mm^3	Conc. of EDTA Na_3 uM/ml	Cell Count With Agent After Incubation Cells/mm^3	PMA Without Agent %	PMA With Agent %
1200	600	5	800	50	33.3
1200	600	5	800	50	33.3
1200	600	10	900	50	25.0
1200	600	10	900	50	25.0
1200	600	15	900	50	25.0
1200	600	15	900	50	25.0
1200	600	20	900	50	25.0
1200	600	20	900	50	25.0

Trisodium EDTA shows, at concentrations of 10 micromoles per ml and above, a fifty per cent decrease in adherence of cells.

TABLE 22C

Cell Count Without Agent Before Incubation Cells/mm^3	Cell Count Without Agent After Incubation Cells/mm^3	Conc. of EDTA Na_4 uM/ml	Cell Count With Agent After Incubation Cells/mm^3	PMA Without Agent %	PMA With Agent %
1000	700	5	700	30	30
1000	700	5	700	30	30
1000	600	10	700	40	30
1000	600	10	700	40	30
1000	600	15	800	40	20
1000	700	15	800	30	20
1000	700	20	800	30	20
1000	700	20	800	30	20
1000	700	25	900	30	10
1000	700	25	900	30	10

Tetrasodium EDTA prevents adherence of cells better at higher concentrations than at low concentrations. Twenty five micromoles per ml gives an approximately 30% reduction in adherence, whereas twenty micromoles and below, gives a 15% reduction in adherence.

TABLE 22D

Cell Count Without Agent Before Incubation	Cell Count Without Agent After Incubation	Conc. of EDTA K_2	Cell Count With Agent After Incubation	PMA Without Agent	PMA With Agent
Cells/mm^3	Cells/mm^3	uM/ml	Cells/mm^3	%	%
1200	600	5	700	50	41,7
1200	600	5	700	50	41,7
1200	600	10	800	50	33,3
1200	600	10	800	50	33,3
1200	600	15	1000	50	16,7
1200	600	15	1000	50	16,7
1200	500	20	1000	58,3	16,7
1200	600	20	1000	50	16,7

Dipotassium EDTA acts best at a concentration of 15 uM/ml and above and prevents the adherence of approximately 70% cells.

TABLE 22E

Comparison of the Various EDTA's

EDTA	Conc. of uM/ml	PMA
Na_2	5	21,4
Na_3	5	33,3
Na_4	5	30,0
K_2	5	41,7
Na_2	10	23,1
Na_3	10	25,0
Na_4	10	30,0
K_2	10	33,3
Na_2	15	15,4
Na_3	15	25,0
Na_4	15	20,0
K_2	15	16,7

This table compares the efficiency of the various EDTA's at similar concentrations. The disodium salt is clearly the most efficient at preventing adherence and the dipotassium salt is only slightly less efficient, but only at a concentration of 15 uM/ml. The trisodium salt and the tetrasodium salts are the least efficient.

3. Effect of Supernatants from Cultured Lymphocytes Pulsed with PHA.

Varying volumes of MAF containing supernatants from cultured lymphocytes pulsed with PHA (as described in Part 1.), were added to mouse macrophage cells in suspension and the tubes incubated for three hours. To some of the tubes, EDTA Na_2 was added to give a final concentration of 10uM. All tubes were made to the same final volume by the addition of PBS. Controls had no supernatant added. The results are shown in Table 23A.

Table 23B shows the effect of supernatants from different lymphocyte cultures. 10ul of supernatant was added to macrophage cells in suspension and containing 10uM EDTA Na_2. Four different supernatants were used.

In Tables 23A and 23B, PMA was not used, but the equation for PMA is modified to show the change that occurs in macrophage adherence on addition of MAF containing supernatants.

$$\text{Percentage Change in Macrophage Adherence (PCMA)} = \frac{\text{Mean No. of Non-Adherent Cells (Control).} - \text{Mean No. of Non-Adherent Cells with MAF (Test).}}{\text{Mean No. of Non-Adherent Cells.}} \times 100$$

TABLE 23A

Cell Count Before Incubation	$EDTA\ Na_2$	Volume Supernatant	Cell Count After Incubation	PCMA
$Cells/mm^3$	uM/ml	ul	$Cells/mm^3$	%
a. 1300	None	None	600	-
a. 1300	None	None	600	-
b. 1300	10	None	1100	-
b. 1300	10	None	1100	-
c. 1300	None	10	400	33,3
c. 1300	None	10	400	33,3
d. 1300	10	5	600	45,5
d. 1300	10	5	600	45,5
e. 1300	10	10	600	45,5
e. 1300	10	10	600	45,5
f. 1300	10	15	600	45,5
f. 1300	10	15	600	45,5
g. 1300	10	20	600	45,5
g. 1300	10	20	600	45,5

Varying the volume of supernatant does not affect the PCMA.

Note: The tubes (b.) containing EDTA alone are taken as control in calculating the PCMA of d. to g. This is done because the difference between cell counts of the tubes with no EDTA and those containing supernatant is lower than the difference between those containing EDTA and those containing EDTA plus supernatant.

In c., tube a. is taken as control.

TABLE 23B

Supernatant	Cell Count After 3 Hr. Incubation	PCMA
ul	Cells/mm^3	%
None	1000	-
None	1000	-
1	500	50
1	500	50
2	500	50
2	500	50
3	500	50
3	500	50
4	500	50
4	500	50

There is no difference in PCMA when MAF containing supernatants from different lymphocyte cultures is used.

DISCUSSION

As was mentioned in the introduction, at present, the methods used for measuring macrophage adherence are laborious and time consuming. The method which is employed here, makes use of an electronic method for counting the macrophage cells, left in the supernatant of a suspension of cells, after allowing adherence to take place on the walls of the tube. As can be seen from Experiment 1., the adherence of the cells to the walls of the tube, takes place naturally, without the addition of any inhibiting or activating agents. This adherence is time dependent and is more constant after three hours, than after one or two hours. It can be noted here, that as the Sysmex microcellcounter only counts in increments of one hundred cells per mm^3, one unit difference in the count can produce as much as a ten per cent co-efficient of variation in the adherence. This variation could probably be decreased if many more macrophage cells were used, but this would involve the sacrifice of many mice for one experiment which would not be practical for routine use. The macrophage cells from different mice compared to cells from one mouse has little effect on the PMA.

It can be seen from Table 20B, that the higher the number of cells per tube, the more the adherence is. This is most likely due to the fact that macrophage cells in vitro do 'adhere' to one another and the more cells

that there are, the more chance of this mutual adherence (Alleroyce et al., 1979). Because of this effect, it is important that for each experiment, the number of cells in each tube is kept constant.

Chelating agents are extensively used to prevent macrophage and/or monocyte spreading and adherence (Garvin et al., 1961 ; Ackerman & Douglas, 1978 ; Wood et al., 1979). All these workers employ the disodium salt of EDTA, which binds both calcium and magnesium ions, and it can be seen from Table 21E, that the disodium salt is more efficient in preventing adherence than the other EDTA salts used. It is not surprising that the dipotassium salt is of similar effectiveness, as in both these salts, there are two free acidic groups which bind the calcium and magnesium ions better than acidic groups which are associated with sodium or potassium ions. It can be seen, that the trisodium and tetrasodium salts are progressively less efficient, owing to the decrease in acidic groups. As the dipotassium salt is slightly less efficient, especially at lower concentrations than the disodium salt, and as potassium is a larger atom than sodium, it may be that there is a steric hindrance within the molecule that decreases the chelating power of the dipotassium salt. It would be interesting to try the dilithium salt of EDTA, to see if this explanation of steric hindrance is valid. Other substances that

could be used are: ethyleneglycol - bis (B- aminoethyl ether) N,N' - tetraacetic acid (EGTA), which selectively binds calcium (Rabonowitz, 1964), but this substance is reported to be less effective than EDTA (Ackerman & Douglas, 1978). Other specific calcium antagonists could also be used, such as 8 - (N,N - diethylamine) - octyl - 3,4,5 - trimethoxy benzoate hydrochloride (Chiou & Mamondi 1975), or lanthanum chloride (Rosenberger & Triggle, 1978).

The last experiments show the effect of MAF containing supernatants on macrophage adherence. MAF causes significant adherence in the presence of EDTA Na_2. This adherence is the same as if neither EDTA nor MAF is added to the cells. The results show that the difference between the control using no EDTA and the test using no EDTA is small, therefore it is better to use the difference between the control using EDTA and the test using MAF and EDTA which is larger. These figures give a larger PCMA and should be more accurate and reproducible in measuring macrophage adherence.

Like LIF, MAF shows optimum activity at low concentrations and its activity does not change upon increasing the amount. As MAF is active in the presence of EDTA, it may be that adherence of macrophage cells in the presence of MAF is partially independent on the presence of calcium and/or magnesium ions. It is impossible from this experiment, to say what is happening here as there is slightly more adherence in the absence of EDTA, than in its presence.

In conclusion, this type of system for detecting macrophage adherence, does work and hopefully can be used to study aspects of macrophage adherence, such as mechanism of action and the effect of many substances, as well as MAF on it.

PART 3.

Introduction

LIF is reported to be a protein of 68 000 molecular weight (Rocklin, 1975), whereas MAF has not been characterised completely (Kotkes & Pick, 1979) and is probably the same as migration inhibiting factor (MIF) (David & Remold, 1976 ; Cohen & Yoshida, 1979). MIF has been characterised as a heat stable (56°C for 30 minutes), 25 000 MW protein (Rocklin et al., 1972). On the other hand, MAF has been reported to have a molecular weight of 25 000 - 65 000 daltons (Remold & David, 1971 ; Nathan et al., 1973). Weisser et al., (1981) reported that there are at least three MIF's produced from lymphocytes stimulated with Con A, with the following molecular weights: 23 000, 65 000 and one, an apparent molecular weight of 23 - 43 000. Sorg & Geigy, (1976) state that MIF may occur in various states of polymerization or aggregation, which makes characterisation by classified biochemical procedures difficult (Geczy et al., 1980).

This seems to suggest that there is more than one substance with MAF properties. Because of these facts, it was decided to try and separate by gel filtration, the LIF and MAF activity from the supernatants from stimulated lymphocyte culture.

The experimental section is divided into two sections, section one deals with LIF and MAF produced from PHA

stimulated lymphocytes and section two, with their production from the mixed lymphocyte reaction.

SECTION 1.

METHOD

1. Two millilitres of supernatant was obtained from PHA pulse stimulated lymphocytes as described in Part 1.

2. The proteins in the combined supernatants were precipitated by bringing the supernatants to 70% saturation with ammonium sulphate, followed by stirring for 12 hours at 4°C. The resultant suspension was centrifuged at 10 000g for 20 minutes and the precipitate redissolved in 20ml of 0,05M acetate buffer pH 5,0 (Georgiades et al., 1979). This solution was dialysed against 4 litres of the same buffer (two changes) for 24 hours.

3. The dialysate was concentrated to 20ml, using a Minicon M15 concentrator and 10ml of this fractionated on a previously prepared Sephadex G-100 column (90 X 1,5cm) using the acetate buffer as the eluting solution. The flow rate was 6ml/hour and 3ml fractions were collected. The fractions were pooled as described in the results section and concentrated to 1ml, using a Minicon M15 concentrator.

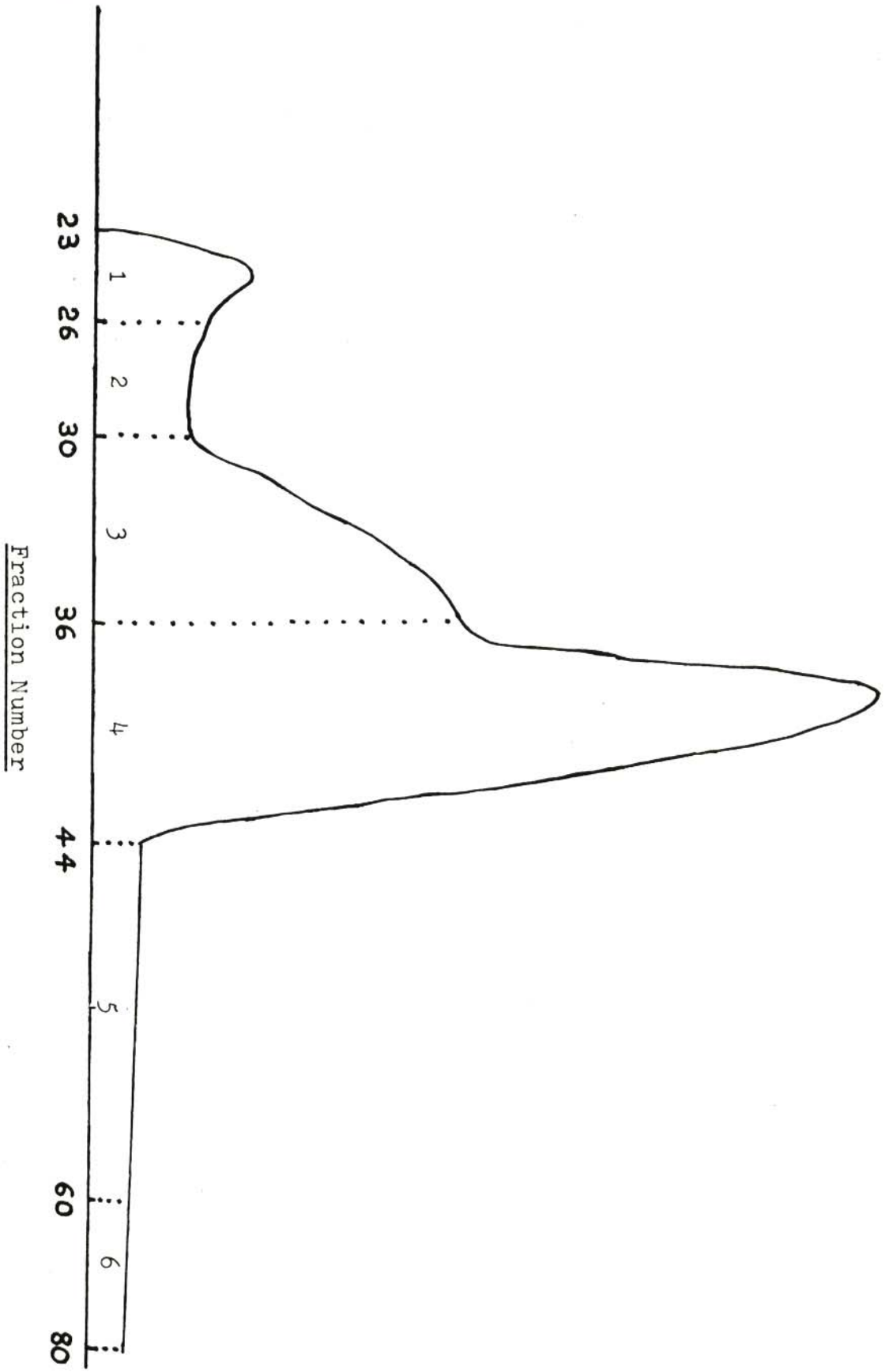
4. Aliquots from Steps 1. and 2. (above), as well as the pooled fractions were tested for LIF and MAF activity, as described previously.

A total of 80 aliquots of 3ml fractions was collected as shown in Figure 3 and pooled into six fractions as also shown.

Fraction 1. was pooled from tubes 23 to 26.
Fraction 2. was pooled from tubes 25 to 30.
Fraction 3. was pooled from tubes 31 to 36.
Fraction 4. was pooled from tubes 37 to 44.
Fraction 5. was pooled from tubes 45 to 60.
Fraction 6. was pooled from tubes 61 to 80.

The large peak shown in the figure is due to albumin which was present in the culture medium and the other peaks are due to other serum proteins. The lymphokines, being present in very small quantities are not detected in the elution spectrum shown.

Fig. 3.



Assay for LIF Activity.

Supernatant	Radius of Migratory Area		M.I.
	Test	Control	
Step 1.	5,0	7,5	0,67
Step 2.	5,0	7,5	0,67
Fraction 1.	8,0	8,0	-
Fraction 2.	6,0	6,0	-
Fraction 3.	5,0	7,5	0,67
Fraction 4.	5,5	7,0	0,79
Fraction 5.	7,0	7,0	-
Fraction 6.	6,0	6,0	-

These results show that LIF activity is only present in Fractions 3 & 4, which corresponds to the large protein peak shown in Figure 3. There is no loss in LIF activity caused by the purification processes as the LIF activity in the fractions is comparable with the LIF activity immediately after culture.

Assay for MAF Activity.

Supernatants	Cell Count After 3 Hour Incubation	PCMA %
None	1500	-
None	1500	-
Step 1.	500	66,6
Step 2.	500	66,6
Fraction 1.	1500	-
Fraction 1.	1500	-
Fraction 2.	700	53,3
Fraction 2.	700	53,3
Fraction 3.	500	66,6
Fraction 3.	500	66,6
Fraction 4.	700	72,0
Fraction 4.	700	72,0
Fraction 5.	700	72,0
Fraction 5.	700	72,0
Fraction 6.	1400	6,6
Fraction 6.	1500	-

MAF activity is present in Fractions 2,3,4 & 5. Fraction 6 shows very little if any, activity. There is no loss in MAF activity during the purification process.

MAF activity is present in the large protein peak shown in Figure 4. as well as in the shoulder between this peak and the first protein peak, but is not present in the first protein peak.

SECTION 2.

METHOD

1. Fifty millilitres of supernatant was obtained from the mixed lymphocyte reaction as described in Part 1. The cells were cultured for four days.

2. The proteins in the combined supernatants were precipitated by bringing the supernatants to 70% saturation with ammonium sulphate, followed by stirring for 12 hours at $4^{o}C$. The resultant suspension was centrifuged at 10 000g for 20 minutes and the precipitate redissolved in 20ml of 0,05M acetate buffer pH 5,0 (Georgiades et al., 1979). This solution was dialysed against 4 litres of the same buffer (two changes) for 24 hours.

3. The dialysate was concentrated to 1ml using a Minicon M15 concentrator, then fractionated on Sephadex G-100, as described in Part 3, Section 1.

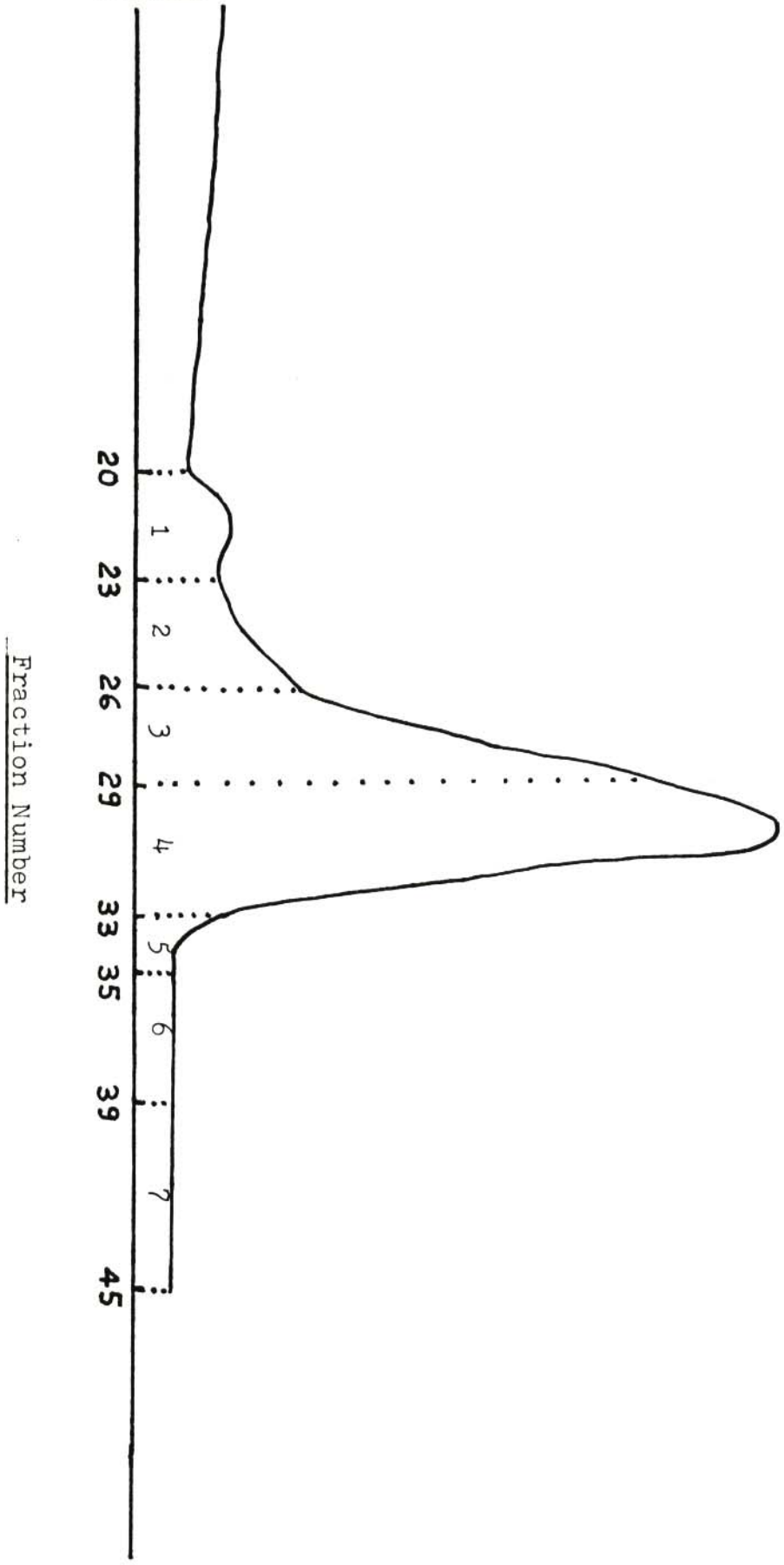
4. Aliquots from Steps 1. and 2. (above), as well as the pooled fractions were tested for LIF and MAF activity, as described previously.

A total of 45 aliquots of 3ml fractions was collected as shown in Figure 4 and pooled into seven fractions as also shown.

Fraction 1. was pooled from tubes 20 to 23.
Fraction 2. was pooled from tubes 24 to 26.
Fraction 3. was pooled from tubes 27 to 29.
Fraction 4. was pooled from tubes 30 to 33.
Fraction 5. was pooled from tubes 34 to 35.
Fraction 6. was pooled from tubes 36 to 39.
Fraction 7. was pooled from tubes 40 to 45.

The peaks shown in the figure are again due to the presence of serum proteins which were present in the culture medium.

Fig. 4.



Assay for LIF Activity.

Supernatant	Radius of Migratory Area		M.I.
	Test	Control	
Step 1.	2,5	10,0	0,25
Step 2.	4,0	10,5	0,38
Fraction 1.	10,0	10,0	-
Fraction 2.	11,0	11,0	-
Fraction 3.	10,0	10,0	-
Fraction 4.	10,5	10,5	-
Fraction 5.	10,0	10,0	-
Fraction 6.	6,0	10,0	0,60
Fraction 7.	10,5	10,5	-

These results show that LIF activity is only present in Fraction 6. There is no peak associated with this fraction. A loss in LIF activity occurred during the purification process.

Assay for MAF Activity.

Supernatant	Cell Count After 3 Hour Incubation	PCMA %
None	1300	-
None	1300	-
Step 1.	400	69,2
Step 2.	400	69,2
Fraction 1.	1300	-
Fraction 1.	1300	-
Fraction 2.	400	69,2
Fraction 2.	400	69,2
Fraction 3.	400	69,2
Fraction 3.	400	69,2
Fraction 4.	500	61,5
Fraction 4.	500	61,5
Fraction 5.	1300	-
Fraction 5.	1300	-
Fraction 6.	1300	-
Fraction 6.	1300	-
Fraction 7.	1300	-
Fraction 7.	1300	-

The results show that MAF activity is only present in Fractions 2,3 & 4 and there is no loss of activity during the purification procedures.

DISCUSSION

The first section shows that using Sephadex G100, MAF activity can be separated from LIF activity, but not vice versa. This would suggest that MAF is not a single substance as far as promoting macrophage cell adherence is concerned. It could be that LIF has MAF activity, again in promoting macrophage adherence. As MAF activity is found in fractions collected before LIF activity is found, this suggests that there is an MAF with a larger molecular weight than LIF. If the results of Sorg & Geczy, (1976) are correct and MIF is the same molecular species as MAF, there may be an aggregate molecule of MAF with a molecular weight of 12 500 (Geczy et al., 1980), giving an MAF molecule larger than LIF. This large molecular weight MAF could be in agreement with the molecular weight of MIF of 82 000, reported by Dumonde et al., (1972). It is possible that the disparity of these results may be due to the conditions of lymphocyte activation and the methods used in the purification of the molecules.

These conclusions about the molecular weights do not agree with the figures in the introduction as reported by Rocklin (1975) and Remold & David, (1971). On the other hand, MAF activity is shown in Fraction 5. which shows that at least one MAF has a molecular weight of less than that of LIF which is in agreement with the above reported results.

The second section gives completely different results from those shown in the first section, in that LIF activity and MAF activity were completely separated. The MAF produced is present in the same fractions as in the first section, therefore it can be concluded that the same species of MAF are produced in both methods of stimulation. LIF, on the other hand, was only present in Fraction 6., which gives it a much lower molecular weight than the LIF produced by PHA stimulation of lymphocytes. Possible explanations for this are that LIF is like MAF and exists as aggregate molecules, these aggregate molecules being produced from lymphocytes under PHA stimulation and not from the MLR. It could be that during the purification procedure, LIF aggregates are split into LIF monomers.

This difference in LIF produced from the differing stimulatory methods was suggested in Part 1. and these results help to confirm that the LIF from PHA stimulation is different from that produced from the MLR.

As can be seen from the results, the LIF activity decreased during the purification process and this may again point to changes in the LIF aggregate molecules (if they do exist).

It is impossible from the experimental results given here, to conclude exactly, the nature of the LIF molecule but the results do point to the fact that this compound is an aggregate molecule and the degree of polymerisation depends on the method of preparation, and it may be that

the activity of LIF varies with its degree of polymerisation.

In conclusion, it can be stated that stimulated lymphocytes produce more than one MAF species which can promote macrophage adherence. Some of these molecules can be separated from LIF, but using this system of separation, LIF activity cannot be separated from MAF activity when lymphocytes are stimulated with PHA but the activities can be separated when lymphocytes are stimulated by the MLR.

CONCLUSION

Lymphocytes stimulated by the mitogenic substances, phytohaemagglutinin (PHA), Pokeweed mitogen (PWM) and Concanavalin A (Con A), as well as those lymphocytes stimulated during the mixed lymphocyte reaction (MLR), produce lymphokines which affect 1. Polymorphonuclear cell migration under agarose. 2. The adherence of mouse peritoneal macrophage cells to plastic surfaces. These lymphokines are termed 1. Leucocyte inhibitory factor (LIF) and 2. Macrophage activating factor (MAF).

The measurement of LIF by the agarose plate method was found to be a very sensitive test, although many factors such as pH, type of agarose and presence of HEPES affect the migration of PMN cells. The migration inhibition of PMN cells is not suitable for the quantitative assay of LIF, but is suitable for qualitative assays. Using this test, it was found that the LIF produced from the MLR was different to LIF produced from PHA stimulated lymphocytes, owing to their differing effects on the migration of PMN's. This was confirmed by carrying out gel filtration on LIF containing supernatants from PHA stimulated lymphocytes and from the MLR. The LIF from both types of cultures was determined to be different with respect to their molecular weights. It is also possible that B-lymphocytes produce an LIF which is different to that produced from T-lymphocytes, as stimulation of these cells with mitogens other than PHA, produced an LIF active substance with a differing effect on

PMN migration.

The method given for the measurement of MAF activity proves to be a quick and reliable procedure, like the agarose plate method. It is very sensitive and should prove to be a valuable tool in further studies on MAF.

In both methods of lymphocyte stimulation, the spectrum of MAF activity is similar, each showing three MAF fractions. These MAF containing fractions could be one molecule in various degrees of polymerisation.

There is still much to be done on the molecular nature of lymphokines, such as LIF and MAF and it is hoped that newer techniques will become available to purify and precisely define the lymphokines in chemical terms.

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