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A STUDY OF DRUG EFFECTS ON IN-VITRO RESPONSE IN DELAYED HYPERSENSITIVITY WITH PARTICULAR REFERENCE TO MACROPHAGE MIGRATION INHIBITION AND THE ROLE OF PROSTAGLANDINS

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1980

TO

Bill and Bet

"Stimulate the phagocytes ...."

Sir Ralph Bloomfield-Bonington in

"The Doctor's Dilemma" by George Bernard Shaw

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ABSTRACT:
A STUDY OF THE EFFECTS OF PHARMACOLOGICALLY ACTIVE AGENTS ON REACTIONS OF DELAYED HYPERSENSITIVITY WITH PARTICULAR REFERENCE TO THE ROLE OF PROSTAGLANDINS AND THE MACROPHAGE MIGRATION INHIBITION ASSAY.

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1. The purpose of this thesis has been to study the interactions of pharmacologically active materials possibly involved in chronic immuno/inflammatory reactions. In particular the effects of lymphokines on in vitro guinea-pig peritoneal cell migration and the interaction of these mediators of cellular immunity with histamine, glucocorticosteroids and prostaglandins.
2. Both the human polymorphonuclear leucocyte cell migration test and the guinea-pig peritoneal cell migration inhibition test(MMI) have been standardised and the time course of cell emigration monitored. Differences between antigen and mitogen effects were shown; the time course of MIF generation has been studied and the optimal times for measurement delineated.
3. The generation and use of lymphokine(LK) standards have been studied and the reproducibility of standard MMI responses demonstrated. Using standard LK's MIF activity has been shown to be heterogeneous in relation to lymphocyte transforming(MF) and vascular permeability increasing(IVP) LK activities. These results demonstrate the heterogeneity of LK's and indicate that several LK activities should be monitored when assessing cellular immune function.
4. The ability of histamine to inhibit antigen induced MMI has been demonstrated. Neither histamine H_1 nor H_2 receptor antagonists were able to block the histamine induced response. These results imply the possibility that histamine may modify MIF activity by a mechanism not involving the H_1 or H_2 receptor sites.
5. Three glucocorticosteroids have been shown to enhance the migration of guinea-pig cells in vitro and to inhibit MMI. These results imply that the steroids modify cell movement directly providing a physiological rather than a pharmacological antagonism.
6. E-type prostaglandins have been shown to inhibit the generation of MIF, MF and to enhance vascular permeability LK activity. The demonstration that inflammatory macrophages generate PGE_2 in response to LK has led to the proposition that E-type PG's act as modulators of LK generation.

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Plan of cell migration chamber.

ABBREVIATIONS

pg:	picogramme
ng:	nanogramme
ug:	microgramme
mg:	milligramme
g:	gramme
kg:	kilogramme
ul:	microlitre
ml:	millilitre
l:	litre
uM:	micromolar
mM:	millimolar
M:	molar
mm:	millimetre
cm:	centimetre
sq.mm:	square millimetre
sec:	second
min:	minute
h:	hour
MW:	molecular weight
i.v.:	intravenous
i.p.:	intraperitoneal
i.d.:	intradermal
s.c.:	sub-cutaneous
iu:	international unit
nCi:	nanocurie
uCi:	microcurie
mCi:	millicurie
^{3}H:	tritium
^{125}I:	iodine isotope ^{125}
dpm:	disintegrations per minute
C:	degrees Celsius
G:	guage
g:	force of gravity
<:	less than
>:	greater than

SECTION 1: INTRODUCTION

Inflammation as a response to injury has been recognised and studied for almost as long as records are available, from the civilisations of the Tigres Euphrates Basin (circa 2000 BC) and the Greek and Roman Empires where Celsus, in the first years after the birth of Christ, described the cardinal signs of inflammation as turgor, rubor and calor.

The inflammatory response to foreign organisms or tissue damage exhibits a wide range of symptoms both in terms of the kinetics and the cellular pathology of the lesions.

Minor injury leads to a mild inflammatory response composed primarily of hyperaemia and fluid exudation with a little infiltration of leucocytes. Such lesions disappear fairly rapidly and it is the more severe inflammatory responses with major infiltration of leucocytes and tissue monocytes that have the potential to develop into the granulomatous lesions associated with the chronic response (that is a persistent, cellular and often destructive lesion). The major phenomena associated with the chronic inflammatory response are the presence of long lived mononuclear cells, proliferation of lymphoid cells and the presence of large numbers of epitheliod cells and fibroblasts (Zweifach, Grant and McCluskey, 1974).

It is the chronic granulomatous inflammatory lesion that has been most widely associated with the delayed type hypersensitivity response both in terms of the cellular infiltrates and disease processes.

1.i) DELAYED HYPERSENSITIVITY AND ITS RELEVANCE TO THE PATHOLOGY OF CHRONIC INFLAMMATION

The earliest associations between the inflammatory response and immunological phenomena described by Jenner (1798) and Kock (1890) were based upon studies of the effects of injection of extracts from vaccinia virus or tubercle baccilli and as a result of this the chronic inflammatory reactions evoked in recipients became known as bacterial allergies and subsequently delayed type hypersensitivity.

The classic examples of chronic granulomatous inflammation include tuberculosis, syphilis and leprosy due to invasive organisms; sarcoidosis and rheumatoid arthritis of unknown aetiology and banal inflammatory lesions such as the dermatoses. All of these conditions have been associated with the delayed hypersensitivity response and thus whilst it is premature to assume too much it could be argued that all chronic inflammatory conditions contain a delayed hypersensitivity component, although the converse is not true, for example allergies to poison ivy or chemicals (Raffel, 1961).

The distinction between the so-called immediate and the delayed type hypersensitivity responses was formally made by Zinsser in 1925 and has led to the modern characterisation of the anaphylactic immediate skin response with a peak of erythema and oedema within thirty minutes whilst the delayed dermal response to bacterial and other allergens commences 4-5 hours after challenge and reaches a maximum at 24-28 hours.

1.ii) DELAYED HYPERSENSITIVITY AND CELL MEDIATED IMMUNITY (CMI)

Attempts to explain the delayed hypersensitivity response in chronic inflammation stretch back to the early twentieth century and early work was concentrated on attempting passive transfer of delayed hypersensitivity so as to clarify the mechanisms underlying the response. In 1909 Helmholtz claimed to have achieved passive transfer of the tuberculin skin response by the injection into insensitive guinea-pigs of defibrinated blood from animals heavily infected with tuberculosis. Bail in 1910 also claimed transfer of tuberculin sensitivity by injecting a broth of infected organs into normal guinea-pigs and during the next thirty five years many attempts were made to transfer delayed hypersensitivity in both man and animals using tissue extracts (Bail, 1910), sera (Zinsser & Mueller, 1925), cell free filtrates (Lange, 1924) and scrapings from skin test sites (Fellner, 1919).

Attempts at transfer with serum were generally negative (Freund, 1926; Hanks, 1935) and Dienes and Schoenheit in 1926 showed conclusively in guinea-pigs that serum would transfer an immediate type hypersensitivity to egg white as measured by skin test, complement fixation and precipitation reactions but that the delayed response to tuberculin could not be transferred by serum injected in large volumes intracardially, atopically or into the skin (Dienes & Schoenheit, 1926 & 1927). However, these workers considered that the different responses still had the same underlying mechanism, that is the stimulation of antibody formation. In 1942 Landesteiner and Chase demonstrated that living peritoneal exudate cells (PEC) could transfer the delayed response to picryl chloride from sensitive guinea-pigs to naive animals. The response was marked and the transfers were consistently reproducible and in 1945 Chase demonstrated that viable lymph node cells, spleen cells and paraffin oil-induced peritoneal exudate cells from tuberculin sensitive donor guinea-pigs could confer a delayed response to old tuberculin in recipient animals. If the cells used for transfer were heated at 48^{o}C for 15 minutes, or frozen and thawed, or if the cells were removed from the inoculum then recipients failed to react to old tuberculin. During the rest of that decade many workers repeated the pioneer work of Chase (Cummings, Hoyt & Gottshall, 1947; Kircheimer and Weiser, 1947; Kircheimer, Weiser and Van Liew, 1949; Metaxas & Metaxas-Buhler, 1948). In 1951 Haxthausen was able to transfer contact sensitivity

to dinitrochlorobenzene using buffy coat white cells from guinea-pig blood, but not with a predominantly polymorphonuclear leucocyte saline-induced peritoneal exudate. In 1949 Lawrence first demonstrated that peripheral blood white cells in man injected intradermally could transfer tuberculin sensitivity as measured by skin testing on another site in the arm. All the subjects had been previously shown to be tuberculin negative and the induced sensitivity lasted from 1 week to longer than two months (Lawrence, 1959).

Since the 1940's the numbers of immune responses shown to be transferable with viable cells has been greatly enlarged and cell transfer experiments have led to some understanding of the delayed hypersensitivity and cellular immune responses. Thus transfer of contact sensitivity to chemicals has been shown to be specific (Chase, 1953), transfer can be made between strains of animals (Bauer & Stone, 1961), but not between species (Chase, 1953; Rosenthal, Litt & Baer, 1955) and in 1954 Mitchison involved cell mediated responses in transplantation immunity by demonstrating faster tumour graft rejection in mice which had received grafts of lymph-nodes from donors showing cellular reactions to such tumours.

1.iii) CELL MEDIATED IMMUNITY IN VITRO: CELL MIGRATION INHIBITION

Whilst much useful information has been gained from the in vivo studies described in the previous section, major advances in the understanding of cellular immune responses have also come from the development of in vitro models for the cellular interactions during delayed hypersensitivity responses in vivo.

Following the experimental analysis of anaphylaxis by use of isolated smooth muscle preparations, Rich and Lewis in 1932 decided to attempt an investigation of the allergic reaction in vitro. They considered that cells in plasma (during their migration in culture) must come into contact with, for instance, tuberculo-protein added to the plasma and that the behaviour of these cells subsequent to such contact could provide information about the allergic response in vivo. The cells used were either washed buffy coat cells or splenic tissue explants from tuberculous guinea-pigs. When old tuberculin was placed in culture at concentrations that did not affect normal cells the migration of cells from tuberculin sensitive animals was severely restricted (Rich & Lewis, 1932). Rich and Lewis took this to indicate cell damage or death and noted that it was the migrating polymorphonuclear and 'monocyteclasmatocyte' cells which were affected but not the fixed tissue cells or lymphocytes, finally they argued against the response being due to free antibody.

The results obtained by Rich and Lewis were also noted by Aronson who additionally differentiated the response to tuberculin in vitro from the anaphylactic type response of cells from animals sensitised to horse serum (Aronson, 1931). Aronson, however, like Rich and Lewis, considered that the response was due to specific cytolysis of the tissue culture cells by antigen.

Since 1932 several other studies have been undertaken using explants as indicators of tuberculin sensitivity (Heilman, 1963; Carpenter, 1963). Moen and Swift had demonstrated in 1936 that the tuberculin response was antigen specific using streptococcal and tuberculin infected guinea-pigs. The same workers showed that the macrophages were the cell type

most affected and quantitated the response by direct comparison of explants in the presence and absence of antigen (Moen & Swift, 1936).

Much of the early work stressed the cytotoxic effects of tuberculin (Heilman, Feldman and Mann, 1944; Miller & Favour, 1951), however, by the 1950's there was a large body of evidence demonstrating that specific inhibition of tissue explant migration was not dependent on the death of the responding cells (reviewed by O'Neill & Favour, 1955). For example, oxygen consumption of peritoneal exudate cells showed no change when sensitised cells were exposed to tuberculin (Marks & James, 1953), similarly lysozyme release by human leukocytes in tissue culture was unaffected by tuberculin (Kerby & Chauduri, 1953).

The explant system of in vitro cell migration inhibition is still being successfully employed by Johanovsky and his colleagues (Pekarek & Krejci, 1974). These workers have confirmed the inhibitory effects of tuberculin in the absence of cell death (Svejcar & Johanovsky, 1961) and have used this technique to study a wide range of problems associated with in vitro tests of cellular immunity including culture conditions (Svejcar & Johanovsky, 1965; Svejcar, Johanovsky & Pekarek, 1967a), mediator actions (Svejcar, Pekarek & Johanovsky, 1969) and the effects of pharmacologically active agents including histamine and serotonin on the migration inhibition response (Svejcar, Pekarek & Johanovsky, 1967b).

The technique of packing inflammatory cell populations into capillary tubes was introduced in 1962 by George and Vaughan and has proved extremely useful as a quantitative in vitro test for the inhibitory effects on cell movement of antigens causing delayed hypersensitivity responses. These workers washed out guinea-pig peritoneal exudate cells (PEC) five days after intra-peritoneal injection of mineral oil. The cells were then packed into capillary tubes and allowed to migrate out into culture medium containing homologous serum. As with the explant techniques the peritoneal exudate cells obtained from the animals previously sensistised to tuberculin were inhibited from migrating from the capillary tubes when the culture medium contained a purified protein derivative of old tuberculin (PPD). George and Vaughan were able to show a dose related inhibition of cell migration although their main concern was in demonstrating the differences between antibody mediated responses and the delayed responses

evoked by tuberculin in animals sensitised to egg albumin. Egg albumin sensitised animals showed immediate type skin test reactivity and PEC were not inhibited from migrating whereas tuberculin sensitised animals had a delayed skin response and demonstrated migration inhibition. As well as the technical improvement provided by the capillary tube technique this system had the additional advantages of firstly, using the cell population most active for passive transfer of delayed hypersensitivity in vivo (Bloom & Chase, 1967) and, secondly, such peritoneal exudate cell populations included the two most active cell types involved in cell-mediated immune responses, the lymphocyte and the macrophage. The system developed by George and Vaughan has been extensively used since 1962 in essentially the same form as originally described although a number of minor modifications of technique have inevitably developed amongst different groups of workers (Bray & Morley, 1977). An early modification of the technique was to add the antigen to the surrounding culture medium alone rather than add it to the cell suspension prior to packing the cells, thus allowing several different antigens to be tested on a single cell population (David, Al Askari, Lawrence & Thomas, 1964a). Cell migration inhibition was demonstrated to be antigen specific as peritoneal exudate cells from animals sensitised to ovalbumin whilst responding to ovalbumin in vitro failed to show migration inhibition in the presence of an unrelated antigen, diptheria toxoid (David et al., 1964a). The response to such materials confirmed that inhibition of cell migration was caused by antigens eliciting delayed hypersensitivity responses rather than via the generation of antibody as animals sensitised to diptheria or ovalbumin showed no demonstrable circulating antibodies to these materials (Uhr, Salvin & Pappenheimer, 1957). Both the antigen specificity of the migration inhibition response and the differentiation from antibody mediated responses have been further confirmed and extended. Thus cells from guinea-pigs sensitised by tuberculin exhibited cell migration inhibition to tuberculin PPD prior to the appearance of detectable anti-PPD antibody (Ricci, Romagnani, Passaler & Billioti, 1969). Cell migration inhibition was demonstrated to be specific to the carrier protein of 2-4 dinitrophenol-guinea-pig albumin hapten protein conjugates (David, Lawrence & Thomas, 1964b), in agreement with in vivo experiments which showed that the delayed hypersensitivity response was dependent upon the protein carrier

whereas antibodies were demonstrated to be largely hapten specific (Benaceraff & Levine, 1962). Under certain conditions antibody has been demonstrated in supernatants from sensitised lymphocytes cultured in the presence of antigen (Meyers, Shoji, Haber, Remold & David, 1972), so that the possible involvement of antibody in some in vitro cellular immune responses cannot be ruled out.

Whilst cell migration inhibition correlates well with delayed hypersensitivity skin responses in vivo other immunological agents have been reported to be able to cause inhibition. For example, cytophilic antibody may cause migration inhibition via formation of antigen/antibody complexes (Amos, Gurner, Olds and Coombs, 1967; Spitler & Fudenberg, 1970).

There are several studies assessing the possible involvement of complement in the cell migration inhibition response to antigen. Cell migration inhibition occurs in the absence of serum or in the presence of serum heated to 56°C indicating that complement does not play a role in the response. Complement derived from the cells in culture may of course play a role although inhibition still occurs if cells are cultured in the presence of specific complement inhibitors such as the C3 factor inhibitor cobra venom factor (David & David, 1972).

1.iv) THE CONCEPT OF SOLUBLE MEDIATORS OF CELL MEDIATED IMMUNITY: MIGRATION INHIBITION FACTOR (MIF)

Early experiments using the capillary tube technique showed that as few as 2.5% of sensitised cells in a mixed peritoneal exudate cell population were enough to cause the whole cell population to respond to the specific antigen (David, Lawrence & Thomas, 1964c; Bloom & Bennett, 1968). Such experiments also demonstrated that the sensitive cell populations needed to be viable for the transfer of the inhibitory response to a non-sensitised cell population (David et al., 1964c). Purified macrophage populations showed little or no migration inhibitory activity in the presence of specific antigen, however, lymphocyte rich populations derived from guinea-pig lymph nodes when added to such purified macrophage populations at concentrations as low as 1% lymphocytes were able to stimulate migration inhibition activity in response to specific antigen (Bloom and Bennett, 1966). These results implied that the lymphocyte was the initiator of the response and that the macrophage was the target cell. Such studies further implied that there was a transfer of information from lymphocyte to target cell, a possibility also suggested by Svejcar and his colleagues who attempted to transfer the capacity for cell migration inhibition from one spleen fragment to another in tissue culture (Svejcar, Johanovsky and Pekarek, 1967c). Studies using metabolic inhibitors demonstrated that the migration inhibitory response necessitated metabolic activity on the part of the sensitised cell population. Thus the addition of the proteolytic enzyme trypsin to sensitised lymphocyte populations inhibited the ability of such cells to transfer the capacity for migration inhibition to unsensitised peritoneal exudate cells (David et al., 1964b). Such trypsinised lymphocyte populations retained the capacity to transfer delayed hypersensitivity and further regained their capacity to cause cell migration inhibition after 24 hours culture in the absence of trypsin (David et al., 1964b).

These results led to the suggestion that the sensitised lymphoid cell population secreted a mediator able to modify the cellular response to antigen in delayed hypersensitivity. Such a mediator had previously been postulated by Marks and James by analogy with the release of

histamine from basophils during immediate type hypersensitivity responses (Marks & James, 1953). This suggestion had led to the demonstration of an active material known as lymph node permeability factor released from homogenised lymph nodes which was capable of causing increased vascular permeability (Willoughby, Boughton & Schild, 1963; Schild & Willoughby. 1967). Such a mediator was considered tobe preformed in the lymph node, however, the evidence from the cell migration inhibition studies suggested that lymphocyte populations were capable of generating new materials in response to specific antigen challenge.

In 1966 David and, independently, Bloom and Bennett demonstrated that supernatants from sensitised guinea-pig lymphocytes following culture with specific antigen contained materials capable of causing cell migration inhibition when added to non-sensitised peritoneal exudate cell populations (David, 1966; Bloom & Bennett, 1966). Bloom and Bennett incubated PEC from tuberculin sensitive guinea-pigs with PPD and after 24 hours supernatants contained materials able to cause inhibition of the migration of macrophages derived from the peritoneal cavity of unsensitised animals. In similar experiments David incubated lymph node lymphocytes from animals sensitised to OCB-BCG or ovalbumin with the respective antigens. The supernatants from such cultures caused macrophage migration inhibition when incubated with unsensitised PEC. Both groups of workers were able to demonstrate that supernatants from cells incubated without antigen or with an unrelated antigen contained no migration inhibitory factor (MIF) and these experiments provided the first clear evidence that cellular immune reactions could be mediated via soluble products derived from lymphocytes activated by specific antigen.

Since 1966 the presence of MIF like activity has been demonstrated in a wide range of animalspecies including mice (Al Askari, David, Lawrence & Thomas, 1965), rabbits (Svejcar et al., 1967c), man(Søberg & Bendixen, 1967) and invertebrates (Prendergast & Suzuki, 1970). The inhibitory activity was found not to be exclusive to macrophages, thus human peripheral blood neutrophils and human lymph-node cells may react to MIF (Thor & Dray, 1968). This lymphocyte material also exhibited some cross-species reactivity as tuberculin sensitised human lymphocytes in the presence of PPD when mixed with normal guinea-pig macrophages caused inhibition of the

macrophage population (Rajapakse & Glynn, 1970), and MIF from human lymphocyte populations when concentrated, caused inhibition of guinea-pig PEC (Thor, Jureziz, Veach, Miller & Dray, 1968).

A variety of cell sources have been utilised to generate MIF activity including PEC (Bloom & Bennett, 1966), lymph node cells (David, 1966), peripheral blood leucocytes (Bartfeld & Kelly, 1968; Thor et al., 1968; Dimitriu, Thompson & Bona, 1974), and spleen cells (Svejcar et al., 1967a).

A wide variety of antigens have been shown to stimulate MIF production ranging from artificial hapten-protein conjugates (David, 1966), and synthetic polypeptides (David & Schlossman, 1968) to viral and parasitic antigens such as mumps (Feinstone, Beachey & Rytel, 1969), and leishmania (Bryceson, Bray, Wolstencroft & Dumonde, 1970). Tumour cells have also been shown to stimulate MIF production in the guinea-pig (Kronman, Wepsic, Churchill, Zbar, Boros & Rapp, 1969). Physico-chemical characterisation has revealed a number of relatively clear properties. The activity is associated with high molecular weight material precipitated by a 40-90% saturated ammonium sulphate solution. However, attempts to determine the molecular weight of MIF have proved difficult with the majority of studies putting the molecular weight within the range of 35,000 - 100,000 daltons (Remold & David, 1971; Rocklin, Remold & David, 1972). Guinea-pig MIF activity has an electrophoretic mobility associated with albumin or pre-albumin and is stable after heating to 56°C for 30 minutes (Remold, Katz, Haber & David, 1970; Rocklin et al., 1972). Biochemical characterisation has shown that MIF generation is associated with active protein synthesis as the protein synthesis inhibitor puromycin inhibits MIF production by antigen stimulated guinea-pig lymph node cells (David, 1955). Trypsin treatment abolishes antigen induced migration inhibition (David, et al., 1964b), however, there are varied reports of its action on MIF with Bennett and Bloom (1967) showing that trypsin treated lymphocytes fail to generate MIF, a report not confirmed by Bartfeld and Atoynaten (1971), who found that trypsin inhibits the action of MIF on macrophages rather than the production of MIF by sensitised lymphocytes.

The question of the antigen dependency of MIF was first raised by Svejcar and his colleagues who demonstrated MIF activity in rabbit lymph node lymphocyte supernatants only when small amounts of the inducing antigen (PPD) were present (Svejcar, et al., 1967c). This work was extended by

Amos and Lachmann, using insoluble PPP-polyaminostyrene as antigen and removing the antigen from the culture supernatants by centrifugation. These workers found that the supernatants only caused migration inhibition when the antigen was reintroduced (Amos & Lachmann, 1970). Such results have led to the description of an antigen dependent MIF activity in mice (Hochva, Krejci, Pekarek, Svejcar & Johanovsky, 1975), rabbits (Svejcar, Pekarek & Johanovsky, 1969) and sheep (Lowe & Lachmann, 1974), thus there appears to be different activities associated with migration inhibition, some being antigen dependent and some active in the absence of antigen.

MIF activity in vitro has been demonstrated in man to a wide variety of antigens and in many disease states since the initial demonstrations of human MIF activity by Bendixen and Søberg (1967) using human periphal blood white cells in the capillary tube test system. MIF activity has been demonstrated in man to autologous mammary tumour extracts (Andersen, Bjerrum, Bendixen, Schiødt & Dissing, 1970), in patients demonstrating auto-immune symptoms including Hashimoto's thyroiditis (Søberg & Halberg, 1968), multiple sclerosis (Bartfeld & Atoynaten, 1970), Sjogrens syndrome (Søberg & Bertram, 1968), Crohns disease (Dykes, 1970) and glomerulonephritis (Zabriskie, Lewshenia, Moller, Wehle and Falk, 1970). Humans with delayed hypersensitivity to extrinsic antigens such as pollen also have demonstrable MIF activity (Brostoff & Roitt, 1969). The leucocyte migration inhibition test has been used to demonstrate a cellular immune response to human conditions ranging from microbial and parasitic to chemically induced, and including drug allergies; auto-immune conditions; tumours and transplantations illustrating the diversity of the cellular immune response (Table 1).

TABLE 1 DISEASES AND CONDITIONS ASSOCIATED WITH CELL MEDIATED IMMUNITY

Viral infections

- Lymphocytic choriomeningitis virus
- Mumps
- Pox viruses
- Tobacco mosaic virus
- Vaccinia

Bacterial infections

- Brucellosis
- Leprosy
- Listeria
- Staphlococcal disease
- Tuberculosis

Fungal infections

Parasitic infections

- Cutaneous leishmaniasis
- Schistosomiasis
- S. American Trypanosomiasis

Anergy

- Candidiasis
- Hodgkin's disease
- Sarcoidosis

- Addison's disease
- Contact sensitivity
- Cystic fibrosis
- Encephalitis
- Glomerulonephritis
- Granuloma
- Hashimoto's thyroiditis
- Multiple sclerosis
- Myaesthenia gravis
- Peripheral neuropathies (Guillain-Barrè syndrome)
- Rheumatoid arthritis
- Systemic lupus erythematosis
- Transplantation immunity
- Tumour immunity
- Viral tumour

- Drug hypersensitivity

1. v) LYMPHOKINES

Since the demonstration of MIF in 1966 a very large number of antigen specific lymphocyte derived factors have been described and given the generic term lymphokines (Dumonde, Wolstencroft, Panayi, Matthew, Morley & Howson, 1969). The properties of such lymphocyte activation products include the ability to stimulate thymidine incorporation into non-sensitised lymphocytes (mitogenic), to stimulate directional movement in macrophages and polymorphs (chemotaxis), to directly kill target tumour cells in culture (cytotoxic) and to cause a 2-4 hour weal and flare reaction when injected into the skin of a non-sensitised animal (inflammatory). Lymphocytes from a variety of animal species are capable of generating lymphokine activity and these lymphocyte activation products modify a wide range of cell functions in vitro and in vivo (Table 2).

Since the demonstration of a variety of different properties present in lymphocyte culture fluids many attempts have been made to characterise the active materials and to determine whether or not the range of properties could be ascribed to a single or several different molecular species. There now exists considerable biochemical evidence for the view that lymphokines are a heterogeneous population of materials probably glycoproteins with a molecular weight range of 12,000 - 100,000 daltons (David, 1973; Bray & Morley, 1977), however, the purification procedures described are complex and result in a considerable loss of total activity making subsequent bioassay more difficult. The problems of purification have made it difficult to distinguish between some lymphokine activities such as, for instance, guinea-pig migration inhibition factor from mitogenic or inflammatory activities derived from the same culture supernatants. Indeed, recent evidence suggests considerable heterogeneity even between MIF or LIF activities from the same species (Remold and Mednis, 1977; Weiser, Greineder and David, 1979). The introduction of a standard preparation to provide a reference material for comparison with other lymphokine activities would provide a procedure whereby different lymphokine preparations may be distinguished on the basis of their relative potencies. Using multiple point parallel line bioassay it would then be possible to make a quantitative comparison of the potency of lymphokine materials in a similar manner to the analysis used by Black and his

TABLE 2 SOME ACTIVITIES OF LYMPHOKINES

Factor	Target cell	Action
IN VITRO		
Migration inhibitory (MIF)	Macrophage	Inhibits movement
Macrophage aggregating (MAF)	Macrophage	Enhances aggregation
Macrophage chemotactic (MCF)	Macrophage	Causes directional movement
Macrophage slowing	Macrophage	Reduces electrophoretic movement
Macrophage arming	Macrophage	Stimulates cytotoxicity
Macrophage mitogenic (MMF)	Macrophage	Stimulates proliferation
Morphological	Macrophage	Various (eg rounding up)
Macrophage activating	Macrophage	Increased metabolism (e.g. enhanced cAMP release)
Mitogenic or blastogenic (MF)	Lymphocyte	Increased thymidine uptake
Transformation potentiating	Lymphocyte	Enhances ongoing transformation
Helper	Lymphocyte	Enhances antibody generation
Chemotactic	Lymphocyte	Causes directional movement
Blast transformation inhibiting	Lymphocyte	Inhibits blast cell formation
Chemotactic	Neutrophil	Causes directional movement
Chemotactic	Eosinophil	Causes directional movement
Leucocyte migration inhibitory (LIF)	Buffy coat leucocytes	Inhibits movement
Osteoclast activating (OAF)	Osteoclasts	Causes bone decalcification
Lymphotoxin	Cell line cultures	Cytotoxic
Proliferation inhibition (PIF)	He La Cells	Inhibits multiplication
Cloning inhibition (CIF)	He La Cells	Inhibits formation of clones
Inteferon	Various cells	Protects against viral infection
IN VIVO		
Macrophage disappearance (MDF)	Macrophage	Removes macrophages from peritoneal exudates
Skin reactive (SRF)	Skin	Causes inflammation
Inflammatory (IF)	Skin	Increases vascular permeability
Lymph node activating	Lymph node paracortex	Activation of the paracortex
Lymph node activating	Lymph node germinal centre	Causes proliferation of germinal centre cells

colleagues when characterising the H_1 and H_2 activities of histamine (Black, Duncan, Durant, Gannelin and Parsons, 1972).

In view of the problems associated with the purification of lymphokines attempts have been made to standardise the preparation and assay procedures and to introduce sources of large amounts of lymphokine activity. For instance, the ability of the plant mitogen PHA to generate lymphokine activity in the absence of antigen provides a source of lymphokine activity not dependant upon the variable antigen sensitivity of the donor lymphocytes and the small number of specifically responding cells. Recently large amounts of lymphokine activity have been generated from bacterial culture supernatants (Yoshida, Cohen, Bigazzi, Kuratsuji & Asmden, 1975) and from transformed lymphocyte cell lines (Yoshida, Kuratsuji, Takada, Minowada & Cohen, 1976), providing a possible source of lymphokine for purification. Whilst the materials generated by PHA stimulation or derived from cell-line culture supernatants exhibit many of the properties of antigen specific lymphokines they may not be the same materials and an examination of the properties of these products with reference to antigen specific lymphokine activity would seem essential. The cell migration inhibition assay has been widely used and provides a useful test for comparing lymphokine like activities, however, the practise of comparing the area of cell migration inhibition after a fixed time in culture severely limits the usefulness of this assay. An examination of the inhibitory activities of lymphokine-like materials throughout the culture period would be of much greater analytical value. The time-course of cell migration may be followed relatively easily by the use of time-lapse cinematography and could thus provide information on the kinetics of cell migration inhibition in response to specific antigen, non-specific lymphocyte activators such as PHA, lymphokines and other materials with the capacity to cause cell migration inhibition.

1. vi) LYMPHOKINES AS MEDIATORS OF DELAYED HYPERSENSITIVITY AND INFLAMMATION

The range of responses evoked by the lymphocyte activation products makes them potentially of critical importance in cellular immune pathology and chronic inflammation. However, many of the properties of lymphokines have only been demonstrated in vitro and their role as pharmacological mediators of chronic inflammatory processes has not been established. An application to lymphokines of the criteria proposed by Dale in 1929 for an adequate definition of a pharmacological mediator provides an indication of the extent to which these materials may be regarded as mediators of cellular immunity and by extension delayed hypersensivity in vivo. Criteria for establishing that lymphokines act as mediators of cellular immune responses would ideally include:

1 Evidence that these materials can induce a delayed hypersensivity response in vivo.

2 Evidence that lymphokines are generated, released and metabolised or inactivated during a delayed type hypersensivity response.

3 Evidence that inhibition or specific antagonism of lymphokine activity reduces or abolishes a delayed hypersensivity reaction.

a) INDUCTION OF A DELAYED HYPERSENSIVITY RESPONSE IN VIVO

There is considerable in vitro evidence that lymphokines may modify cell movement and function, for example, they are chemotactic for both monocytes and polymorphs, they inhibit the migration of monocytes, stimulate cell differentiation and division and may be directly cytotoxic (Table 2). All of these phenomena could help to explain the pathology of a delayed hypersensivity lesion, however, in vivo evidence of lymphokine activity would be more convincing. Injection of lymphokines into the guinea-pig skin causes erythema (Bennett & Bloom, 1968) and increased vascular permeability with a peak response after 4-5 hours (Morley & Williams, 1973). Histological analysis of such lymphokine induced skin lesions has shown that in contrast to the mononuclear cell rich antigen induced lesion, the

predominant infiltrating cells are polymorphonuclear leucocytes (Franco, Kelly & Morley, 1978). Intralymphatic injection of guinea-pig lymphokine preparations causes both germinal centre proliferation and activation of the paracortex in the draining lymph nodes (Kelly & Wolstencroft, 1974; Kelly, Wolstencroft, Dumonde & Balfour, 1972). Following injection of partially purified lymphokine into the rabbit knee joint a chronic synovitis develops with some of the symptoms of human rheumatoid arthritis such as the accumulation of lymphocytes and macrophages into the synovial tissue (Andreis, Stastny & Ziff, 1974).

Injection of a specific antigen into the guinea-pig peritoneal cavity containing glycogen induced peritoneal exudate cells leads to a marked reduction (90%) in the macrophage content of these exudates (Nelson & Boyden, 1963). This macrophage disappearance reaction (MDR) can be passively transferred to non-immunised recipients by lymphocytes from sensitised donors (Sonozaki & Cohen, 1971). Such a response indicates that the MDR may represent an in vivo model of delayed hypersensivity paralleled by the in vitro models of macrophage aggregation and migration inhibition.

Further evidence for such a proposition has recently been provided.Thus, following antigen challenge MIF activity has been detected in the 25-50000 MW fractions of guinea-pig peritoneal cavity fluid following partial purification of the fluid over a Sephadex G100 fraction column (Van Maarsseveen, 1977).

b) RELEASE OF LYMPHOKINES DURING AN INFLAMMATORY RESPONSE

Lymphokine like activity has been detected following both experimental and pathological inflammatory responses. As mentioned above, following injection of antigen into the peritoneal cavity of guinea-pigs sensitised to that antigen there is an inflammatory response involving the emigration of lymphocytes and macrophages into the peritoneal cavity resulting in the generation and release of MIF activity (Van Maarsseveen, 1977).

The presence of lymphokine activity (MIF) has been demonstrated in synovial fluid derived from the joints of animals with experimental arthritis (Stastny, Cooke & Ziff, 1973) and from the joints of patients with classical rheumatoid arthritis (MIF and mitogenic factor; Stastny, Rosenthal, Andreis & Ziff, 1975).

Lymphokine activity has not until recently been convincingly demonstrated in the circulation (Yoshida, Benacerraf, McCluskey & Vassalli, 1969) and it may be that in order to detect activity in vivo the lymphokines must ideally be released into sites such as the joint space or the peritoneal cavity during an inflammatory response where there may be enhanced production and some protection from catabolic enzyme activity. Recent attempts to induce lymphokine activity in sera by intra-muscular injections of BCG in guinea-pigs followed by intravenous challenge with antigen have met with some success (Yoshida & Cohen, 1974). MIF activity could be detected in the sera from immunised animals and the activity was maximal 6-12 hours following challenge. The time of maximal MIF activity parallels the times of greatest production of circulating monocytes following intravenous antigen challenge in sensitised animals (Willoughby, Coote & Spector, 1967), a response reflecting the cellular immune status of the animal. Very recently lymphokine activities have been measured in the circulation of mice following intravenous sensitisation and challenge with considerable variation in the activities measured between different strains of mice (Salvin & Neta, 1979). Very little is known about the inactivation or metabolism of lymphokines and elucidation of such problems will have to await the purification of these putative mediators coupled with the application of sensitive bioassay procedures.

c) INHIBITION OF LYMPHOKINE ACTIVITY

A veriety of materials have been shown to modify lymphokine activity *in vitro* , although many of the drugs or enzymes used may be expected to have other effects as well as inhibition of lymphokine secretion when used *in vivo*. Examples of drugs which modify the effect of lymphokines *in vitro* include the microtubular disruptive drugs such as colchicine and vinblastine (Pick & Abrahamer, 1973) which block the response of macrophages to preformed MIF. Similarly inhibitors of RNA and DNA synthesis such as puromycin or cyclohexamide will block MIF activity at doses below those thought to be toxic to the responding macrophages (Pick & Manheimer, 1974).

The most specific example of inhibition of lymphokine activity *in vitro* has been described by Remold who demonstrated that the sugar α-L-fucose modified the ability of MIF to inhibit the migration of guinea-pig-macrophages (Remold, 1973). The inhibition of MIF activity could also be blocked if the target macrophages were pre-incubated with the enzyme α-L-fucosidase. Remold concluded from this study that there was a requirement for α-L-fucose on the macrophage surface and that the sugar may form part of a MIF binding site. This work has since been extended by Rocklin who has demonstrated a requirement for α-L-fucose on the surface of human monocytes for them to respond to human MIF whilst there is a requirement for N-acetyl-D-glucosamine for the response of human polymorphonuclear leucocytes to human LIF (Rocklin, 1976).

None of the materials tested *in vitro* have been shown to be specific inhibitors of lymphokine activity *in vivo* nor to cause specific reduction of the delayed-type hypersensitivity reaction.

Thus the proposition that lymphokines may be regarded as mediators of cell mediated immunity, delayed hypersensitivity and chronic inflammation *in vivo* has so far not been fully substantiated.

The properties of these materials both *in vitro* and *in vivo* do, however, indicate that they are capable of modifying a variety of cellular functions (see Table 2) and thus provide a group of substances of considerable potential importance in cellular immunity and by extension human disease processes.

1. vii) MODIFICATION OF LYMPHOKINE GENERATION AND ACTION BY ENDOGENOUS MEDIATORS OR MODULATORS OF THE INFLAMMATORY RESPONSE

The interaction with lymphokines of materials either known to be present during the inflammatory response such as histamine or prostaglandins or known to modify inflammatory reactions such as the steroids may provide further insights into the possible role of lymphokines and cellular immunity in inflammatory reactions.

Histamine and the prostaglandins provide examples of the range of materials active in inflammatory lesions which have been proposed to act both as mediators and modulators of cellular responses, whilst the steroids provide a group of drugs both naturally occurring in the circulation as physiological mediators and in wide use as anti-inflammatory agents.

1. viii) HISTAMINE AND INFLAMMATION

The involvement of endogenous histamine in the inflammatory response has been widely documented (Zweifach, et al., 1974) and this material satisfies many of the criteria of a putative inflammatory mediator.

a) INDUCTION OF THE SYMPTOMS OF INFLAMMATION

In 1913 Eppinger noted that injection of histamine into human skin produced a localised oedema (Eppinger, 1913), with later work demonstrating that histamine has powerful agonist actions on vascular permeability and as such contributes to the oedema of acute inflammatory responses (Willoughby, 1973). The major pharmacological properties of histamine are regarded as contraction of smooth muscle, dilatation of small blood vessels and some stimulation of secretions from exocrine glands (e.g. secretion of gastric acids from intestinal parietal cells). This latter property is considered to be of physiological importance (Levine, 1968).

b) RELEASE OF HISTAMINE DURING INFLAMMATION

In mammals histamine is primarily associated with tissue mast cells and blood basophilic leucocytes, both cell types which synthesise and store histamine in cytoplasmic vesicles. Generally, histamine release is associated with mast cell degranulation and release may be stimulated by a variety of substances including proteolytic enzymes (Rocha e Silva, 1956) and compound 48/80 (Paton, 1951). There is considerable evidence that histamine is released after burns, bacterial invasion or chemical injury (Spector & Willoughby, 1968), but the most widely studied mechanism of histamine release is that of the allergic or immediate hypersensivity reaction. After challenge with an antigen eliciting an allergic reaction the response, in man, is the generation of antibodies of several immunoglobulin classes, particularly IgE "anaphylactic" antibodies which bind to the surface of mast cells and basophils and, on contact with antigen, cause the release of histamine via degranulation.

There is little evidence that histamine plays a major role in delayed hypersensitivity reactions or that it is released although there is some indication that there is a basophil rich infiltrate during the early

phase of some delayed hypersensitivity reactions (Dvorak, Dvorak, Simpson, Richeson, Leskowitz and Karnovsky, 1970). There is also evidence that the basophil rich Jones-Mote response recently renamed cutaneous basophil hypersensitivity represents a form of delayed hypersensitivity (Dvorak, 1976). Such reactions may be elicited by proteins in mineral oil and are characterised by a massive infiltration of basophils into the dermis. Lymphocytes and monocytes are also present and there is thus a possibility that these cells may interact with basophils or histamine during the inflammatory reaction.

c) ANTAGONISM OF HISTAMINE AND REDUCTION OF THE INFLAMMATORY RESPONSE

Systemic treatment with the histamine H_1 receptor antagonist mepyramine maleate causes some reduction in the early phases of the inflammatory response to thermal injury although some oedema still occurs (Spector & Willoughby, 1959; Wilhelm & Mason, 1960).

If animals are depleted of stored histamine by pre-treatment with a histamine releasing drug such as compound 48/80 then there is a marked reduction of the increased vascular permeability response to thermal or chemical injury (Spector & Willoughby, 1959), and to fungal infection (Sheldon & Bauer, 1960). In the guinea-pig intra-dermal injection of lymphokine causes a long lasting increase in vascular permeability an early phase of which is inhibited by pre-treatment with the histamine H_1 receptor antagonist mepyramine (Williams & Morley, 1973a).

d) HISTAMINE IN DELAYED HYPERSENSITIVITY

There appears to be little evidence that histamine plays a major role as a mediator in delayed hypersensitivity responses or chronic inflammatory reactions. However, the ability of mepyramine to partially modify the increased vascular permeability due to lymphokine coupled with the effects of histamine on some <u>in vitro</u> T-lymphocyte responses provides a basis for the examination of the effects of histamine antagosists on responses of cellular immunity. Thus, addition of histamine (up to 10^{-5}M) to cultures containing mouse thymus derived lymphocytes will depress the

ability of these cells to lyse target tumour cells following the addition of specific antigen (Plaut, Lichtenstein, Gillespie & Henney, 1973). The histamine is thought to act directly on the lymphocyte via receptors located on the cell surface (Melmon, Bourne, Weinstein & Sela, 1972) and recent evidence suggests that the response occurs via H_2 receptors as the specific H_2 receptor antagonists burimamide and metiamide cause a dose-related reduction in histamine activity in vitro (Plaut, et al., 1973; Lichtenstein & Gillespie, 1973). In earlier experiments involving cell mediated immune reactions in vitro, Svejcar and his colleagues examined the ability of histamine to modify the emigration of spleen cells from cultured guinea-pig spleen fragments and found no significant effect of the drug with doses up to 50ug/ml. These workers were unable to modify the inhibitory effects of specific antigen on spleen cell emigration by the addition of histamine (Svejcar, Pekarek and Johanovsky, 1967b). The negative results in these studies may reflect the speedy metabolism of histamine in culture. The presence of histamine H_2 receptors on T-lymphocytes and the ability of histamine to augment leucocyte cAMP levels in vitro (Bourne, Melmon & Lichtenstein, 1971) suggests a possible role for histamine in the modification of lymphocyte function in vivo.

Finally, Dvorak has speculated that the basophils seen in large numbers during some responses to antigens interact synergistically with T-lymphocytes during such immunological responses with the basophils being short-lived quickly mobilised cells which may then be replaced by tissue mast cells during more chronic inflammatory responses (Colvin & Dvorak, 1974).

1. ix) GLUCOCORTICOSTEROIDS AND INFLAMMATION

Glucocorticosteroids derived from the adrenal cortex are an example of endogenous hormones which have provided medicine with a series of extremely powerful chemotherauputic agents. Corticosteroids are widely used in the treatment of conditions involving inflammatory, immunologic or neoplastic processes (Germuth, 1956; Schwartz, 1968) and whilst many of the results of steroid therapy are well known the mechanisms of action of these powerful hormones has not been fully clarified. In general steroid therapy in man depresses the symptoms of inflammation and immune reactions. Thus topical cortisone reduces the emigration of leucocytes into acute inflammatory skin sites (Rebuck & Mellinger, 1953); intravenous hydrocortisone can cause lymphocytopenia and monocytopenia (Fauci & Dale, 1974); methylprednisolone is able to reduce serum IgG levels (Butler & Rossen, 1973) and steroids will reduce the expression of delayed type skin test reactivity (Bovornkitti, Kangsadal, Sathirapat & Oonsombatti, 1960; Gabriellson & Good, 1967). Because of the wide differences in steroid sensitivity between species it is difficult to relate some experimental work to the highly steroid resistant human. However, the guinea-pig is also steroid resistant and so provides a useful substitute to man in experiments where the in vivo activity of the hormones is to be studied, for instance, steroid treatment may lower tissue levels of histamine in the guinea-pig (Aviado & Carillo, 1970).

Taking into account the differences in species sensitivity to corticosteroids there remains considerable in vitro evidence for the ability of these materials to modify cell function.

a) STEROIDS IN CELLULAR IMMUNITY (TYPE IV HYPERSENSITIVITY)

Treatment of the steroid sensitive mouse with glucocorticosteroids has been shown to inhibit skin allograft rejection (Medawar & Sparrow, 1956). However, much higher doses are needed in the guinea-pig to achieve similar results (Sparrow, 1953). Similarly, in man, high doses of steroids are required to inhibit the response to a tuberculin skin test and the treatment must be given over a prolonged period of 30 days (Germuth, Ottinger & Oyama, 1952).

The behaviour of major cell types involved in cellular immune reactions (T-lymphocytes and macrophages) have been monitored during steroid treatment with evidence of depletion of small lymphoid cells (Claman, 1972) and there have been a number of reports of in vivo steroid inhibition of in vitro lymphocyte blastogenesis in response to PHA or Con-A using both human periphal blood lymphocytes (Zeman, Cohen, Budrys, Williams & Javor, 1972; Fauci & Dale, 1975; Fauci, 1976) and guinea-pig lymphocytes following chronic hydrocortisone treatment (Balow, Hurley & Fauci, 1975). Addition of steroids in vitro will also depress peripheral blood lymphocyte transformation in man (Nowell, 1961; Caron, 1967; Blomgren, 1974).

The activity of steroids on several other supposed T-cell mediated responses to antigen have also been studied thus the cytotoxicity of murine splenic lymphocytes for homologous target cells can be inhibited by cortisol (Rosenau & Moon, 1962); the cytotoxicity of skin allograft sensitised human periphal blood lymphocytes for skin donor fibroblasts may be inhibited by prednisolone (Lundgren, 1970); steroids will inhibit the generation of the lymphokine "lymphotoxin" in man (Williams & Granger, 1969) and in rats, Stavy and his colleagues have demonstrated that hydrocortisone will inhibit sensitised rat lymph node cells from killing mouse fibroblasts via inhibition of the primary activation of the cytolytic mechanism by target cell antigens (Stavy, Cohen & Feldman, 1973). There have been several reports of steroid modification of MIF production and action. For example, injection of prednisolone into tuberculin sensitive rabbits will inhibit subsequent BCG induced cell migration inhibition, however, addition of lymphocytes from sensitised untreated animals to macrophages from treated rabbits will restore normal BCG reactivity implying an action of the steroid on the generation of MIF (Casey & McCall, 1971). A similar conclusion was drawn by Bendtzen who examined the effects of prednisolone on human leucocyte MIF (Bendtzen, 1975), and Wahl and her colleagues in a study of guinea-pig MIF inhibition by hydrocortisone (Wahl, Altman & Rosenstreich, 1975). In contrast to these results two groups have reported that the action of guinea-pig MIF on target macrophages rather than generation of MIF was inhibited by hydrocortisone and dexamethasone (Weston, Claman & Kreuger, 1973; Balow & Rosenthal, 1973). A recent study using rabbits has similarly reported that hydrocortisone

inhibits the action of MIF on target macrophages (Pekerak, Svejcar, Nouza & Johanovsky, 1976). Whilst, in contrast to both of the sets of results reported above, dexamethasone has recently been reported to cause leucocyte migration inhibition in its own right with doses of $0.3 - 1.2 \times 10^{4}$M (Henley, Okas & Aviner, 1976).

There have been many reports of steroid actions on macrophage and monocyte function and in vivo it appears that glucocorticoids act by interfering with the sticking of leucocytes to the vascular endothelium (Allison, Smith & Wood, 1955). In man, both hydrocortisone and prednisolone treatment cause a circulating monocytopenia (Fauci & Dale, 1974; Fauci & Dale, 1975) and injection of cortisone inhibits the infiltration of macrophages into the peritoneal cavity of tuberculin sensitive guinea-pigs challenged intraperitoneally with PPD (Nelson, 1963). Whilst in vitro studies have demonstrated that E.coli induced monocyte chemotaxis and random migration can be impaired by hydrocortisone (Rinehart, Balcerzak, Sagone & Le Buglio, 1974), although in a report dating from 1945 Heilman noted that compound E (hydrocortisone) caused enhanced migration of the large wandering cells from rabbit splenic explants in culture (Heilman, 1945).

From the above it is apparent that glucocorticosteroids exert a wide range of effects on both lymphocyte and macrophage/monocyte function. It therefore seems pertinent to examine the actions of these drugs on in vitro parameters of cellular immunity in order to attempt to clarify their actions both on these cell types in vitro and perhaps shed some light on the mechanisms of their remarkable immunosuppressive potency in vivo.

1. x) PROSTAGLANDINS AND INFLAMMATION

The Prostaglandins (PG's) are 20 carbon fatty acids derived in vivo from polyunsaturated fatty acids and, since their discovery and characterisation from 1930 to the 1960's they have been described in most tissues studied in both man and experimental animals. It seems likely that the prostaglandins play a role in modifying cellular responses in view of the marked capacity of cells and tissues to generate these materials following various forms of stimuli (Ramwell & Shaw, 1970) and the ability of PG's to modify intracellular cyclic 3', 5', adenosine monophosphate (cAMP) levels in a variety of cell types (e.g. activation by PGE's of adenyl cyclase activity in fibroblasts (Peery, Johnson & Pastan, 1971), in contrast to adipose tissue where prostaglandin E_1 (PGE_1) decreases cAMP levels in isolated fat cells (Butcher & Baird, 1968)). In view of the generation of PG's by cells or tissues following stimulation it seems reasonable to examine the inflammatory stimulus as a trigger for PG production and to determine whether prostaglandins may act as mediators or modulators of the inflammatory response. The prostaglandins have been found to fulfil many of the criteria of a mediator of acute inflammatory reactions and there are also indications that they play a role in chronic inflammation and associated humoral and cellular immune response.

a) INDUCTION OF THE SYMPTOMS OF INFLAMMATION

Intradermal injection of E-type prostaglandins in both experimental animals and man causes vasodilatation (Solomon, Juhlin & Kirchenbaum, 1968), erythema (Juhlin & Michaelsson, 1969; Crunkhorn & Willis, 1971) increased vascular permeability and oedema (Crunkhorn & Willis, 1971), all signs of an acute inflammatory reaction. Prostaglandins are able to enhance the oedema caused by other inflammatory agents thus PGE_1 and prostaglandin E_2 (PGE_2) enhance the oedema caused by the subplantar injection of the sulphated polysaccharide, carageenan into the rat hind foot pad (Moncada, Ferriera & Vane, 1973), and increase the vascular permeability response to lymphokine (Williams & Morley, 1973b). Large amounts of PG's have been shown to cause pain (Ferriera, 1972), however, much lower levels of PG's (nanograms) will sensitise pain endings to

other algesic mediators (Ferriera, Moncada & Vane, 1973). Accumulation of polymorphonuclear leucocytes (PMN) may be stimulated by E-type PG's in vitro (Kaley & Weiner, 1971), although the effect seems to be restricted to the rabbit as human and guinea-pig PMN's are unaffected by PG's (Pick & Abrahamer, 1973; Bray & Franco, 1977). Other features possibly relating to chronic inflammatory disease may also be caused by small quantities of PG's, thus stimulation of collagen biosynthesis and an increase in bone resorption have been reported (Blumenkrantz & Sondergaard, 1972; Harris, Jenkins, Bennett & Willis, 1973; Robinson, Tashjian & Levine, 1975). Finally, injection of PGE_1 or PGE_2 into the knee joints of dogs results in a severe arthritis (Rosenthale, Dervinis, Kassarich & Singer, 1972).

b) RELEASE DURING INFLAMMATION

Prostaglandins have been reported to be released following the application of a variety of inflammatory stimuli and from a variety of tissues. Early work concentrated on monitoring ocular inflammation and E-type PG's were shown to be released following manipulation of the eyes of rabbits (Ambache, Kavanagh & Whiting, 1965; Ambache & Brummer, 1968) and during experimental uveitis in the rabbit (Eakins, Whitelocke, Perkins, Bennett & Ungar, 1972). It has since been established that, in man, PG's are released following inflammatory stimuli to the skin such as during contact dermatitis (Greaves, Sondergaard & McDonald Gibson, 1971), or from delayed inflammatory lesions caused by UV irradiation (Sondergaard & Greaves, 1970). Laboratory models of the inflammatory response such as subcutaneous carageenan-induced inflammation or carageenan injection into the paws of rats also stimulate the generation of PG's (mainly PGE_2) (Anderson, Brocklehurst & Willis, 1971; Willis, 1969). Similarly an experimental model for arthritis in rabbits involving the intra-articular injection of adjuvant mineral oil induces PG release (Blackham, Farmer, Radjiwonik & Westwick, 1974). In vitro studies have demonstrated that PG's may be generated following allergic stimulation of the guinea-pig lung (Piper & Vane, 1969). Finally, there is evidence of PG production by rheumatoid tissue both by cultured synovial cells in vitro (Robinson, Smith & Levine, 1973) and in synovial effusions from patients (Higgs, Vane, Hart & Wojtulewski, 1974).

c) INHIBITION OF PROSTAGLANDIN SYNTHESIS AND ITS EFFECTS ON THE INFLAMMATORY RESPONSE

Prostaglandin synthesis may be inhibited by preventing release of substrates (such as arachidonic acid) from cellular storage sites or by inhibition of one or more of the enzymes involved in the PG biosynthetic pathways. There has been a recent proposal that the anti-inflammatory steroids such as hydrocortisone may act via inhibition of phospholipase A2 activity (Nijkamp, Flower, Moncada & Vane, 1976). Many of the anti-inflammatory steroids whilst being apparently ineffective at inhibiting PG synthesis in some systems (Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972), are capable of significant PG synthesis inhibition in isolated cell cultures of guinea-pig macrophages (Bray & Gordon, 1978), so that whilst steroids undoubtedly exert their anti-inflammatory effects in a variety of ways the actions on PG synthesis in vivo are yet to be clarified.

In 1971 Vane and his colleagues demonstrated the ability of several non-steroidal, aspirin-like drugs to inhibit in vitro prostaglandin synthesis (Vane, 1971; Ferriera, Moncada & Vane, 1971; Smith & Willis, 1971). These workers proposed that the aspirin-like non-steroidal anti-inflammatory drugs (NSAID's) acted in vivo via inhibition of PG synthesis. The NSAID's are capable of reducing pain, fever and swelling, all symptoms associated with the formation of PG's thus these results would seem to provide a simple explanation for the mode of action of NSAID's. Some evidence, however, suggests a wider role for the NSAID's in treatment of chronic inflammatory conditions where cellular immune processes may be thought to play a part. Thus, aspirin both in vivo and in high doses in vitro is able to suppress phytohaemagglutinin stimulation of T-lymphocyte thymidine incorporation (Crout, Hepburn & Ritts, 1975; Opelz, Teraski & Hirata, 1973).

The ability of steroids, NSAID's and some other anti-inflammatory drugs to inhibit PG synthesis lends support to the proposition that the PG's are amongst the mediators of both acute and chronic inflammatory responses,

d) PROSTAGLANDINS AS MODULATORS OF INFLAMMATION

There are several precedents which may be drawn upon from other physiological systems to demonstrate a regulatory role for prostaglandins. For instance, PGE_1 suppresses vasoconstriction induced by noradrenaline, angiotensin and vasopressin (Holmes, Horton & Main, 1963; Hedquvist, 1968) and PGE_1 and PGE_2 inhibit the release of noradrenaline from the spleen following sympathetic stimulation. Similarly, PGE_1 reduced lipolysis by isolated adipocytes evoked by adrenaline, adrenocorticotrophic hormone, glucagon and thyroid stimulating hormone (Steinberg, Vaughan, Nestel & Bergstrom, 1963). The action of PGE_1 in adipocytes is thought to be mediated via the enzyme adenylate cyclase (Steinberg & Vaughan, 1967), and such evidence of PG modification of cell function has led to the proposition that PG's may serve as local hormones modifying secretory cell function probably via elevation of cAMP levels following stimulation of adenylate cyclase activity.

E-series prostaglandins are able to modify cyclic-AMP levels in cells known to be involved in the generation of mediators of both anaphylactic Type I hypersensitivity and Type IV delayed type hypersensitivity and so have the capacity to modify immunological reactions during acute and chronic inflammatory processes.

Thus, prostaglandin E_1 is capable of preventing the release of histamine and slow reacting substance of anaphylaxis (SRS-A) from basophils and human lung fragments in vitro (Leichtenstein & De Bernardo, 1971; Orange Austen & Austen, 1971) and consequently may modify Type I hypersensitivity responses. Phagocytic cells involved in Type III immunological reactions are affected by PG's in vitro. Thus, stimulation of human polymorphonuclear leucocyte adenylate-cyclase by E-type PG's leads to increased levels of intracellular cAMP (Bourne et al., 1971) and a reduction of lysosomal enzyme release in vitro (Weissman, Dukor & Zurier, 1971; Weissman, Zurier, Spieler & Goldstein, 1971). Cells thought to mediate Type IV cellular immune responses may also be modified by in vitro treatment with PG's. Thus Henney and his colleagues have demonstrated that stimulation of lymphocyte cAMP levels by PGE_1 leads to a reduction in the in vitro ability of antigen stimulated T-lymphocytes to kill target cell monolayers (Henney, Bourne & Lichtenstein,1972). Prostaglandin modification of lymphocyte c-AMP levels

similarly

parallels a reduction in the incorporation of ^{3}H-thymidine into T-lymphocytes responding in vitro to stimulation by the plant mitogen phytohaemagglutinin (PHA) (Smith, Steiner & Parker, 1971). Furthermore, prostaglandins have been shown to modify some experimental models of inflammatory disease. For example, addition of pharmacologic doses of PGE_1 or PGE_2 into the site of a carageenan induced inflammatory skin response reduces the rate of inflammatory cell accumulation and lysosomal enzyme activity (Zurier, Hoffstein & Weissman, 1973). Such a reduction in cell accumulation could be due to PG modification of chemotaxis as PGE_1 is able to inhibit the leucocyte chemotactic response to bacterial products in vitro (Rivkin & Becker, 1972). In the rat model for arthritis induced by injection of adjuvant pharmacological doses of E-type PG's are capable of supressing the tissue response (Zurier & Quagliata, 1971). Such data coupled with evidence of the later generation of prostaglandins during both carageenan induced inflammation and adjuvant arthritis allows for the supposition that such PG generation may serve to modulate the inflammatory reactions.

The ability of prostaglandins, particularly of the E-series to modify a variety of cell functions and the presence of these fatty acids in a range of inflammatory situations provides a sound basis for a study of the possible interactions between the lymphokines proposed as putative mediators of cellular immune responses and the inflammatory prostaglandins.

1. xi) PURPOSE OF THESIS

1 The in vitro guinea-pig macrophage migration inhibition assay (MMI) will be used to assess the time course of inhibitory activity exhibited by specific antigen acting upon target macrophages derived from sensitised donor guinea-pigs and to compare different antigen (tuberculin or bovine gamma globulin) or mitogen (PHA) responses with a view to providing a standardised assay for lymphokine activity. Similarly, the activity of preformed lymphokines will be compared with culture supernatants purporting to contain MIF activity (Kurloff cell supernatants and lymphoid cell line supernatants) with a view to establishing whether such supernatants exhibit similar kinetics of migration inhibition and may be regarded as acting in the same manner as MIF.

2 The guinea-pig MMI assay test will be used as a parallel line bioassay to assess the relative potencies of a series of similarly prepared lymphokine preparations. Potency ratio estimates for MMI will then be compared by chi-squared analysis to potency ratio estimates of the same lymphokine preparations assayed for mitogenic and inflammatory activities. Such a comparison will allow conclusions to be drawn regarding the degree of heterogeneity present in ostensibly identical lymphokine preparations.

3 The in vitro test of MMI will be used to examine the effects of pharmacologically active drugs upon the secretion and action of the products of antigen stimulated sensitised lymphocytes. Specifically an examination of the possible effects of the endogenous inflammatory mediators histamine and the prostaglandins upon guinea-pig migration inhibition factor.

Similarly, the effects of anti-inflammatory glucocorticosteroids on MMI will be examined as these drugs are known to be active in suppressing aspects of chronic inflammatory lesions and delayed hypersensitivity skin tests. If any of these drugs are found to modify lymphokine activity in terms of MMI then their effects on other tests of lymphokine function such as lymphocyte transformation and increased vascular permeability will be examined.

SECTION 2: METHODS

2 i) ANIMALS

Male and female outbred Hartley guinea-pigs weighing 250 to 500g.

2 ii) IMMUNISATION PROCEDURES

BCG: Glaxo Bacillus Calmette-Guerin (BCG: Glaxo, Greenford, UK) standardised by viable cell count was injected intraperitoneally (i.p.) in 0.1 ml saline. After a minimum of 4 weeks animals were skin tested via intradermal injection of 2 and 20.0ug of a freeze dried preparation of a purified protein derivative of Mycobacterium tuberculosum (PPD: Ministry of Agriculture and Fisheries, Weybridge, UK), dissolved in 0.1ml saline. Animals with erythematous responses of 10mm and over were used in experiments.

BGG/FCA: Animals were immunized by intradermal injection of 200ug bovine-gamma-globulin in saline (BGG: Armour, Eastbourne UK) emulsified with Freunds Complete Adjuvant (FCA: 2mg/ml of freeze-dried tuberculin in Difco Freunds Incomplete Adjuvant: Difco, Detroit, USA) at equal concentrations (v:v) using 4 injection sites per animal into footpads or the shoulders and hips. Intradermal skin tests into flank skin were performed after 3 weeks using 10ug/site BGG in saline with an erythema greater than 10mm being considered positive.

Human tuberculin sensitivity: Human volunteers were skin-tested with 5TUs (1 ug/ml) of a solution of tuberculin PPD (Evans, Liverpool, UK) and skin responses of greater than 5mm of erythema were considered positive.

2 iii) CELL INDUCTION TECHNIQUES

Guinea-pig peritoneal exudate cells were induced by prior injection i.p. of 20ml of a sterile 2% soluble starch solution (amylopectin : Sigma, St. Louis, USA), using a 19G 1½" needle 2 or 3 days prior to sacrifice. Alternatively, 20ml of light paraffin oil B.P. was injected i.p. using a 0 gauge sterile serum needle 3 or 4 days prior to sacrifice.

2 iv) MEDIA

Media used for macrophage migration were Hanks balanced salt solution (BSS) and Eagles Minimal Essential Medium (E-MEM). Hanks BSS was prepared by diluting Hanks stock Medium A and B (Oxoid, London, UK) in a 2.0mM solution of sodium bicarbonate. Eagles MEM stock solution (x 10 concentrate: Flow, Irvine, Scotland) was diluted to one litre with distilled water and 200mM sodium bicarbonate and 200mM glutamine (Flow, Irvine, Scotland) added to give a final concentration of 2.0mM. Benzyl penicillin (sodium) B.P. (Glaxo, Greenford, UK) at 1 x 10^5 iu/l and streptomycin sulphate B.P. (Glaxo, Greenford, UK) at 1 x 10^5 iu/l were included in both Hanks BSS and Eagles MEM. All media were stored at $4^{\circ}C$.

2 v) SERA

Foetal calf serum (FCS: Flow, Irvine, UK) was regularly used as a standard serum. Batches of FCS were obtained and samples tested at concentrations of 5, 10, 20 and 30% against 15% normal guinea-pig serum (NPGS) in the cell migration assay (see also Appendix A3). Batches which did not adversely affect the area of cell migration when compared to NGPS were used at either 10 or 20%. Horse serum (Flow, Irvine, Scotland) was occasionally used and was tested as above. Normal guinea-pig serum was obtained by stunning donor guinea-pigs and bleeding from the heart using a 19G needle. Ten to fifteen ml of blood per animal was regularly obtained. Blood was placed in sterile 50ml glass conical centrifuge tubes and incubated at $37^{\circ}C$ for one hour in a 5% CO_2 in air atmosphere. The clot was detached from the sides of the container to facilitate retraction and the container placed overnight at $4^{\circ}C$. The serum was decanted and centrifuged at 400g for 10 minutes to remove any remaining cells and fibre fragments. All sera were aliquoted into 10ml batches and stored at $-20^{\circ}C$ until used. Sera to be decomplemented were heated for 30 min. at $56^{\circ}C$ in a water bath before freezing.

2 vi) CELL MIGRATION INHIBITION

a) GUINEA-PIG PERITONEAL EXUDATE CELL MIGRATION

Peritoneal exudate cells (PEC) induced by starch or oil injection were harvested from 1 or 2 guinea-pigs 2 to 4 days after injection. Animals were killed by a blow at the base of the neck (cervical dislocation) and shaved ventrally using electric clippers. The peritoneal cavity was opened by a mid-line incision and the cells removed by lavage with 50-100ml of cold (4°C) Hanks BSS. The Hanks, containing peritoneal exudate cells, was placed in sterile 50ml conical glass centrifuge tubes and centrifuged at 400g for 10 min at 4°C (MSE Mistral 4L centrifuge: Measuring and Scientific Equipment (MSE), Crawley, UK). Cells and solutions were then all maintained at 4°C until placed in migration chambers in order to inhibit attachment of PEC to the containers. After centrifugation the supernatants were discarded and the packed cells pooled and resuspended in 10ml of Eagles MEM. Cell viability and numbers were determined by dye-exclusion in a haemocytometer as follows: 0.1ml of the 10ml cell suspension was added to 0.9ml E-MEM and 1.0ml of sterile Eosin solution (8mg/ml in 0.89% saline: BDH, Poole, UK) or Trypan blue (4mg/ml: BDH, Poole, UK) to give a 1:20 cell dilution. Total white cells were counted using a Modified Fuchs Rosenthal haemocytometer (Gelman-Hawksley, Lancing, UK) and all dye-excluding white cells were considered viable. The 10ml cell suspension was centrifuged and re-suspended using E-MEM containing serum to give a concentration of 3 - 5 x 10^7 viable white cells per ml.

Cell migration chambers (Kennedy Chambers: Sterilin, Richmond, UK) were used for macrophage migration inhibition tests (see Appendix B). The sterile 12 chamber (16mm diameter) migration plates were prepared for migration by dispensing inert silicone grease (Gallenkamp, London, UK) from a 2ml syringe onto the upper surface of each chamber and a spot of grease onto the inner, lower surface of the chamber. Test materials were dissolved at appropriate concentrations in E-MEM containing serum and held in 6ml sterile plastic bijous (Sterilin, Richmond, UK) at room temperature until use.

The cells in suspension were taken up by capillary action into 100 ul sterile glass microhaematocrit tubes (A selected plain tubes:

Gelman-Hawksley, Lancing, UK) and one end sealed by a plug of wax. (prepared by melting three parts hard wax (Gallenkamp, London, UK) to one part soft wax (Gallenkamp, London, UK) into 10 cm glass petri dishes to a depth of approximately 4mm).

Plugged capillary tubes containing cells (final concentration 3 - 5 x 10^6 viable cells/tube (See also Appendix A) were placed in 10 ml centrifuge tubes containing a cotton wool plug and spun for 5 min.at 400g in an MSE bench centrifuge (M.S.E., Crawley, UK). Cells and centrifuge were not allowed to become overheated in order to prevent melting the wax plug. Capillaries were spun in batches of 6 with the time between packing the cells into the capillary tubes and their enclosure in the migration chambers minimised as far as possible. Individual capillary tubes were scarified with a diamond pen at the position of the cell/fluid interface and the tube snapped immediately prior to the packed cell pellet being fixed in the migration chambers held by the spot of inert silicone grease. After placing the capillary tube each chamber was immediately filled with appropriate culture medium and a cover-slip used to seal the chamber. The 30mm diameter round glass coverslips (Chance, Smethwick, UK) were stored in alchohol and flame sterilised in a bunsen burner before being placed over the migration chambers with care being taken to ensure that no air bubbles remained inside the chamber. Completed 12 chamber migration plates were numbered, placed horizontally in a plastic storage 'box', and incubated at 37°C in a 5% CO_2 in air atmosphere using an NLTE anhydrous incubator (NLTE, Oldham,UK).

Incubation times varied from 18 to 42 hours.

Following cell emigration from the pellet, images of cell migrations were projected onto Whatman No. 1 filter paper (W.R.Balstom, Maidstone, UK) at a magnification of x 16 using a Gillett and Siebert Analynx light microscope (Gillett & Siebert, London, UK) fitted with a Monocular extension and bench projector unit. The outline of the migration (magnified 16 times) excluding the area of the capillary tube, was recorded manually. Migration outlines were excised and weighed to the nearest milligram using an electric balance (Sartorious-Werke, Scientific Instruments Centre, London UK). One millimeter squares of filter paper were regularly projected and weighed for calibration. Results were

expressed as area of cell migration in square millimetres, or as the migration index employed in immunology calculated by the following formula:

$$\text{Migration Index (MI)} = 1 - \left(\frac{\text{mean control - mean test}}{\text{mean control}} \right)$$

Replicate cell migrations (i.e. a single chamber containing a single pellet of cells) were set up using either a random order obtained from random number tables ('Documenta Geigy' p.101) or balanced and partially balanced designs (Fisher & Yates, 1963). The design of the experiment depended on the numbers of cells obtained and the number of test preparations used.

b) HUMAN PERIPHAL BLOOD LEUCOCYTE MIGRATION

Twenty ml of peripheral blood was taken from a human donor using a 30ml syringe (Sherwood, Crawley, UK), and a 21G needle (Gillett, Isleworth, UK). Two ml of a 6% Dextran solution in saline to give a 0.6% solution in blood (Dextran: Fisons, Loughborough, UK) together with 2ml of a 200 iu/ml preservative and pyrogen-free heparin solution to give 20 iu/ml of blood (Heparin: Evans, Liverpool, UK) were mixed with the blood in the syringe. The syringe was placed vertically in an incubator at 37°C for one hour so that the red cells sedimented towards the plunger, allowing the white cell-rich plasma to be expelled through the needle. A new needle was attached to the syringe after one hour and bent to form an L-shape. The plasma could then be expelled into 10ml sterile glass centrifuge tubes. The white cell-rich plasma was centrifuged at 400g for 10 min at room temperature and the cell pellet resuspended in 10ml of E-MEM for counting and viability, using techniques described in Methods vi)a) above. The cell pellet was resuspended to a concentration of approximately $1 - 2 \times 10^8$ viable cells/ml in E-MEM containing either 10% decomplemented foetal calf serum or 10% horse serum (Flow, Irvine, UK). Migration plates were prepared as in Methods vi) a) and 20 ul Drummond "microcaps" (Drummond: Scientific Supplies, London, UK) were cut in half, sterilised in an oven and used as the migration tubes. Final cell concentration in the tubes was $2 - 4 \times 10^6$ viable cells/tube. Chambers were incubated for 18h and the migration area measured by projection onto filter paper at x 9.6 magnification.

c) TIME LAPSE CINEMATOGRAPHY OF GUINEA-PIG AND HUMAN CELL MIGRATION

Cell suspensions from guinea-pig peritoneal exudates or human periphal blood were prepared as in a) and b) above. Perspex, two-well migration chambers were used and wells were sealed by sterile glass cover-slips attached with inert silicone grease. Cells packed into capillary tubes were placed in the chambers with Eagles MEM containing 15% heat inactivated pooled normal guinea-pig serum for guinea-pig macrophages, or 10% heat inactivated foetal calf serum for human leucocytes.

Chambers were filmed using a Vinten Mark II Scientific Camera (W. Vinten, London, UK) using Kodak plus X reversible 16mm film (Kodak, London, UK). Migrations were illuminated from below by a microscope lamp (Scientific Supplies, London, UK) with three glass heat sinks and a black masking plate placed over the chamber around the migrations to prevent reflected light passing through the perspex surrounds.

Time lapse filming was controlled by a Vinten time lapse control unit set for 15 min exposures and the temperature maintained at 37°C by a thermister placed near the migration plates and attached to two 2KW electric fan heaters via an adjustable control unit.

Film was processed commercially and projected onto a screen for measurement using a Specto 16mm film projector (Specto, Windsor, UK).

Migrations were filmed for up to 65 hours and the results plotted as area of migration versus time.

2 vii) LYMPHOCYTE TRANSFORMATION USING GUINEA-PIG LYMPH NODE CELLS

Normal or immunised Hartley guinea-pigs were killed by cervical dislocation and the axillary, sub-clavian, femoral and popliteal lymph-nodes removed and placed into 9cm sterile Falcon petri dishes containing Hanks BSS. Excess fat attached to the lymph nodes was removed and the nodes excised using a 19G needle. The cell suspension was passed through a sterile fine mesh wire filter (Hemispherical sieve, 120 gauge: Caddish, London, UK) and tissue debris mashed through a course wire filter (80 gauge) with the resulting cell suspension being passed through the fine filter. The cells were centrifuged in an MSE Mistral 4L centrifuge at 400g for 10 min and resuspended in 10 ml of Eagles MEM. Cell counts were performed as for cell migration and the lymphocytes respun and re-suspended at 2 x 10^6 viable cell/ml in Eagles MEM containing 5% pooled normal guinea-pig serum.

One ml aliquots of medium plus serum containing appropriate concentrations of antigen with or without drugs were placed into sterile glass test tubes or sterile 12 x 75mm round bottom Falcon plastic disposable culture tubes (Falcon, Oxnard, USA) and 1 ml of cell suspension added to each tube making a final volume of 2 ml with a cell concentration of 1 x 10^6 viable cells/ml.

Culture tubes were incubated at 37°C with a 5% CO_2 in air atmosphere for 48 hours. Eighteen hours prior to harvest 1 uCi of H^3-thymidine (Radiochemical Centre, Amersham, UK) in 10 ul sterile distilled water was added to each culture tube.

The cultures were harvested by one of two methods:

a) The tubes were cooled to 4°C to stop the reaction and then centrifuged at 800g for 5 min at 4°C. The supernatants were removed and the cells washed once with cold phosphate buffered saline, twice with cold 5% trichloracetic acid (TCA) (B.D.H., London, UK) to precipitate the protein and once with cold methanol to remove any water. In each case the tubes were spun at 800g for 5 minutes and the supernatants discarded. After washing with methanol 0.5ml of 10% v/v hyamine hydroxide (Koch-Light, Colnbrook, UK) was placed in each tube and the tubes incubated in a shaking water bath at 56°C for half an hour in order to solubilise the protein precipitate. After incubation the hyamine solution was

washed into plastic 20 ml disposable scintillation vials (Griffith-Nielson, Billingshurst, UK) by two washes of 7.5ml toluene phospher scinter (Toluene Cocktail P: Fisons, Loughborough, UK). The vials were sealed, placed into a Packard Tricarb Scintillation counter (Hewlett-Packard, Caversham, UK) and counted for 5 min. Background counts were estimated by counting two vials containing 15ml of scinter only. Results were expressed as disintergrations per minute and calculated from the efficiency curve for the counter calculated from channel ratios.

b) Tubes containing the cells were mixed on a Whirlmixer (Whatman GF/A: W.R.Balston, Maidstone, UK) to resuspend the lymphocytes and the 2ml suspensions passed through millipore filters in a Millipore vacuum cell harvester (Millipore (U.K.), London, UK). The tubes were washed with 5ml cold 0.9% saline which was then passed through the filters. Two washes of 5ml 5% TCA and one wash with cold analar methanol followed and the filters were then placed in scintillation vials and dried in a glassware drying oven for a minimum of one hour. After drying, 5ml of Toluene phospher scinter was placed in each vial and the samples counted as in (a).

2 viii) INCREASED VASCULAR PERMEABILITY IN THE GUINEA-PIG SKIN

The assay involved assessing increased vascular permeability by measuring the accumulation of ^{125}I - labelled guinea-pig serum albumin (GPSA) into inflammatory foci in guinea-pig skin. Hartley guinea-pigs (350 - 400 g) were carefully and closely shaved over the dorsal flanks extending from the shoulders to the hips and six injection sites/flank were marked at equal distances on the skin. Animals specifically sensitised to a particular antigen were challenged with that antigen twenty-four hours prior to measurement of the response with ^{125}I - GPSA.

For lymphokine assay normal guinea-pigs were used and the lymphokine injected 2 hours prior to measurement. Animals were injected intradermally in the dorsal flank skin with 0.1ml of the challenge material in 0.9% saline using a 26 gauge needle and tuberculin syringe (Gillette, Isleworth, UK).

^{125}I - labelled guinea-pig serum albumin was prepared from partially purified normal guinea-pig serum (0 - 40% ammonium sulphate precipitation) with the resulting freeze-dried supernatants trace labelled with ^{125}I (Radiochemical Centre, Amersham, UK) with a specific activity of 10 uCi/mg. For injection 1 - 2 uCi ^{125}I - GPSA was mixed with Evans Blue dye in 0.9% saline and animals were injected with 0.5ml of the mixture via the dorsal hind foot vein. After four hours animals were sacrificed by cervical dislocation and one ml of venous blood collected from the jugular.

The dorsal skin was removed including the panniculus carnosus and discs of skin wholly enclosing the lesions were excised using an 11/16" wad punch. Two samples of uninjected skin were also excised as background. The isotope accumulation in the skin and blood samples was measured using an automatic gamma spectrometer.

Three animals were used for each experiment and the thirty six available injection sites arranged in a partially balanced 6 x 6 latin square design developed to minimise inter-site and inter-animal variation (Morley, Wolstencroft and Dumonde, 1973).

The count rates for the skin samples were expressed as ul whole blood equivalent derived from the following equation:

$$\left(\frac{\text{Count rate in lesion - Count rate in uninjected skin}}{\text{Count rate in 1 ml venous blood}} \right) \times 10^3$$

2 ix) GUINEA-PIG CELL CULTURE FOR THE GENERATION OF LYMPHOKINES

Guinea-pig peritoneal exudate cells or lymph node lymphocytes were removed as previously described and suspended in E-MEM containing 5% heat inactivated pooled normal guinea-pig serum to a concentration of 1×10^6 viable cells/ml. Cultures were incubated in Falcon sterile plastic universal bottles (Falcon, Oxnard, USA) or Falcon sterile plastic 20ml flat bottom flasks. Volumes from 10 - 20mls containing antigen and/or drugs were incubated for 24 hours at 37°C in a 5% CO_2 in air atmosphere. After incubation cells were removed by centrifugation at 400g for 10 minutes and the supernatants stored at -20°C prior to purification. Control lymphokine preparations were derived from parallel cell cultures in the absence of antigen or drugs.

2 x) PARTIAL PURIFICATION OF GUINEA-PIG LYMPHOKINES

Lymphokine preparations were concentrated by partial purification using ammonium sulphate precipitation and extensive dialysis. Solid ammonium sulphate (AS) (B.D.H., London, UK) was added to lymphokine supernatants to 40% saturation and the samples mixed at 4°C for a minimum of one hour to equilibrate the solutions. After stirring the samples were centrifuged at 2500g for fifteen minutes and the precipitate discarded. Further AS was added to 90% saturation, stirred overnight at 4°C, centrifuged and the supernatants discarded. The precipitates containing the high molecular weight material concentrated in the 40 - 90% AS fraction were redissolved in a minimal volume of distilled water (2mls) and the solutions transferred into prewetted 18/32 dialysis tubing (Visking, UK) and dialysed overnight against running water then finally for a further 24 hours against cold 0.02 M volatile bicarbonate buffer (1.58 g/l NH_4HCO_3: 0.58 ml/l 2M acetic acid) so as to permit lyophilisation. After dialysis lymphokine rich solutions were placed into round bottomed 100ml

glass flasks and shell frozen by immersion in a mixture of methanol/ solid CO_2. Immediately after freezing the samples were attached to a vacuum freeze drier and dried. Dried preparations were weighed and stored at -20°C.

Precipitation by 0 - 40% AS removed the large molecular weight materials including the inducing antigen, antibody and immune complexes. The extensive dialysis removed salts present in the culture medium. The final preparations of molecular weight material between 10 - 100 thousand contained the bulk of the lymphokine activity. Guinea-pig lymphokines prepared as above have been reported to be stable at -20°C for up to two years. (Bray, Dumonde, Hanson, Morley & Wolstencroft, 1976).

2 xi) GUINEA-PIG PERITONEAL EXUDATE CELL CULTURE FOR PRODUCTION OF PROSTAGLANDINS

Guinea-pig peritoneal inflammatory exudates were induced in the peritoneal cavity as described for cell migration inhibition. Exudate cells were removed by peritoneal lavage with Hanks BSS, washed and counted as for cell migration inhibition. Cell viability was established by the exclusion of eosin - Y or Trypan blue vital dye. Washed peritoneal exudate cells were resuspended in buffered Eagles MEM containing glutamine, antibiotics and 10% heat inactivated foetal calf serum. For routine culture for prostaglandins the cells were incubated at a concentration of 1×10^6 viable cells/ml. 0.5 ml aliquots of the peritoneal exudate cell suspension were dispensed into sterile plastic 12 x 65 mm culture tubes (Falcon, Oxnard, USA) and the cultures were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2 in air. Cultures were generally incubated for 24 hours, after which time the tubes were centrifuged at 400g for five minutes at 4°C and the cell free supernatants removed and stored at -20°C prior to assay for prostaglandin activity.

2 xii) RADIOIMMUNOASSAY FOR E-TYPE PROSTAGLANDIN ACTIVITY

Prostaglandin E-like activity in peritoneal exudate cell culture supernatants was measured by a radioimmunoassay using sheep anti-PGE_2 bovine serum albumin antiserum. The assay

was based on the techniques described by Jaffe (Jaffe, Smith, Newton and Parker, 1971). Tritium labelled PGE_2 (5.0 n Ci/0.1ml; Radiochemical Centre, Amersham, UK) in 0.1 ml aliquots was equilibrated in 2ml polypropylene test tubes (Luckham, Burgess Hill, UK) with 0.1 ml of the unknown sample or standard PGE_2 solution (dose range 0.3 - 40 ng/0.1ml) in 0.1 ml phosphate buffer, pH 7.2 to give a final volume of 0.3 mls. The mixture was mixed well and incubated overnight at 4°C. After incubation 0.1 ml bovine gamma globulin (2.5 mg/0.1 ml) was added as carrier protein and antibody bound labelled PGE_2 was precipitated by addition of 0.4 ml of saturated ammonium sulphate to give a 50% saturated solution. The solutions were mixed and then centrifuged at 1000g for 15 minutes at 4°C. The supernatants were discarded and the precipitate washed once with 0.8 mls 50% ammonium sulphate and then dissolved in 1.2 mls distilled water. The resulting solutions were added to plastic scintillation vials (Koch-Light, Colnbrook, UK) containing 10 mls scintillation fluid (Toluene/Triton X; 2/1 vol/vol, containing 4 g 2, 5 - disphenyloxazole (PPO) and 0.08 g 1, 4 - di - (2 - (4 - methyl) - 5 - phenyloxazolyl) - benzene (DMPOPOP) /litre (Fisons, Loughborough, UK)).

Samples were counted on a Packard 'tri-carb' liquid scintillation counter. Immunoreactive PG-like activity was estimated by interpolation on a standard curve and expressed as PGE_2 equivalent. PGE_2 - BSA antiserum cross reacted 100% with PGE_2; 55% with PGE_1; 11% with 13 - 14 - dihydro - PGE_2; 1.5% with $PGF_{2\alpha}$; 1.2% with 15 - keto - PGE_2; 0.8% with 13, 14 - dihydro - 15 - keto - PGE_2; 0.6% with PGA_2; 0.3% with thromboxane B_2 and 0.2% with PGB_2 (Bray and Gordon, 1978).

2 xiii) STATISTICAL ANALYSIS

Where appropriate a 2-tailed test for significance has been used - the Students t test for parametric data. Values of $p < 0.05$ have been taken to represent a significant difference between two groups of data and in general when a greater degree of significance exists it has not been shown.

For a fuller analysis of statistical methods used for cell migration inhibition see Appendix A b.).

Analysis of varience has been undertaken in several instances (Colquohoun, 1971) and X^2 analysis has been carried out as described in Bray et al., 1976.

During the period of this study the percentage variation of a series of 28 cell migration assays has been calculated. The range of variation (S) was found to be between 7.0% and 24.5% (see also Morley, 1974).

2 xiv) DRUGS

DRUG	FORM	STORAGE	SOURCE
Histamine Acid Phosphate	Crystals	20°C	British Drug Houses (BDH), Poole, UK.
Mepyramine Maleate	"Anthisan" Aqueous solution 50 mg/ml	4°C	May & Baker (M&B) Dagenham, UK.
Semicarbazide Hydrochloride	powder	4°C	BDH
Burimamide	powder	4°C	Smith, Kline & French (SKF), Welwyn Garden City, UK.
Metiamide	powder	4°C	SKF
Hydrocortisone Sodium Phosphate	"Efcortisol" Aqueous solution 100 mg/ml	4°C	Glaxo, Greenford, UK.
Dexamethasone Sodium Phosphate	"Decadron" Aqueous solution 4 mg/ml	4°C	Merck,Sharp &Dohne, (MSD), Hoddesdon, UK.
Prednisolone Sodium Phosphate	"Codelsol" Aqueous solution 16 mg/ml	4°C	MSD
Prostaglandins E_1;E_2; A_1; $F_{2\alpha}$	Solution in 95% Ethanol 1 mg/ml	-20°C	M & B
Indomethacin (milled)	Powder	4°C	MSD

Indomethacin, Burimamide and Metiamide were dissolved in 0.1 N NaOH diluted in aqueous solution and the pH adjusted to pH 7.0 with 0.1N HCl prior to use.

Prostaglandins were diluted in aqueous medium immediately prior to use.

Histamine and semicarbazide were freely soluble in aqueous solution. Stercids were diluted in aqueous solution as required.

SECTION 3: RESULTS

3 i) CELL MIGRATION INHIBITION

Preliminary studies of guinea-pig and human cell migration inhibition have been directed towards clarifying the factors which modify the cellular response in this assay. Variables concerned with the cell collection, preparation and culture conditions, as well as statistical analysis of the results are dealt with in Appendix A. In this section three particular aspects of cell migration inhibition have been investigated in greater depth. Firstly the pattern of cell distribution in the cell population migrating from the capillary tube, secondly the actions of specific antigens or mitogens on cell migration and, thirdly, the time course of guinea-pig and human cell migration in the presence and absence of inhibitory agents.

a) CELL TYPES AND PATTERNS DURING CELL MIGRATION

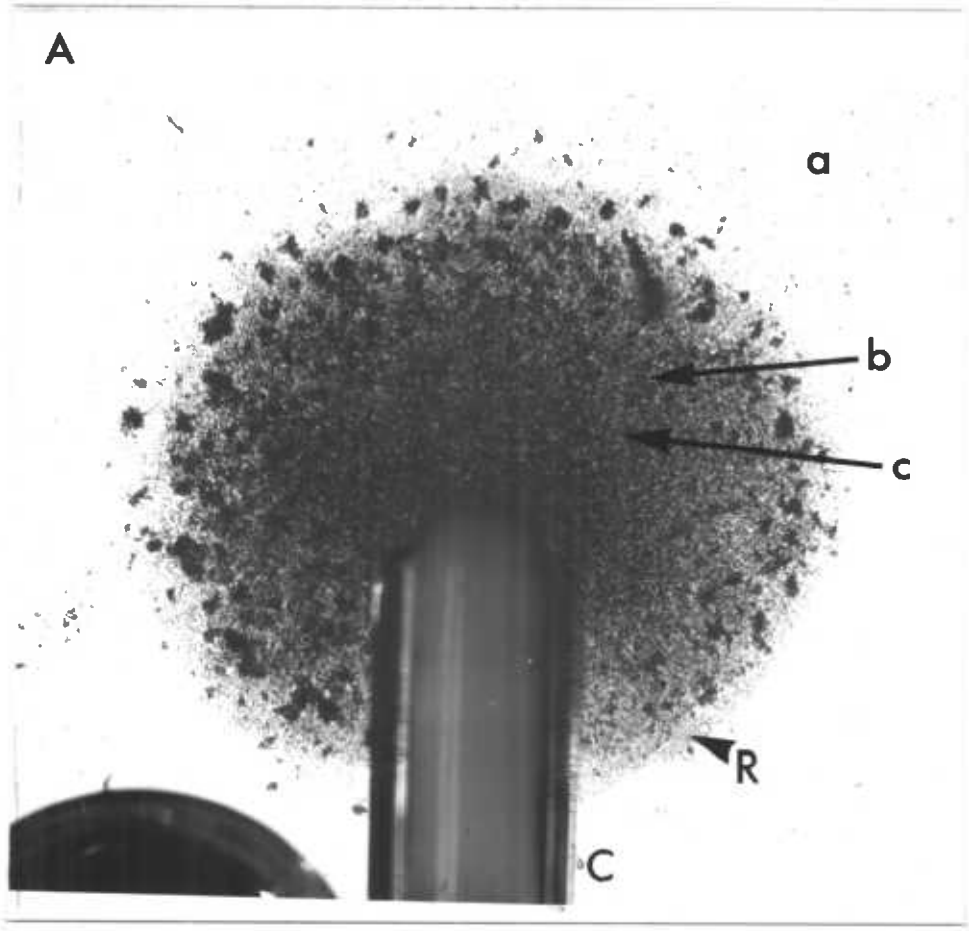
The fans formed by guinea-pig peritoneal exudate cells (PEC) migrating from the capillary tube are shown in Figures 1 and 2. Figure 1A shows the migration of guinea-pig PEC in the absence of inhibitory agents and Figure 1B illustrates the migration inhibition of PEC in the presence of specific antigen after 40 hours incubation. The fans show a relatively regular though not well-defined border and the cells tend to form clumps throughout the area of migration. Groups of cells can be seen beyond the border of the fan and any red cell contamination forms a ring (R) around the border of the exudate cell migration area. Inserts a, b and c in Figure 2 show the cell types present in three different areas of the fan of cells shown in Figure 1A. The first insert (a) is a high power view of an isolated group of macrophages which have migrated beyond the dense cell area.The typical foamy cytoplasm (c) and kidney shaped nucleus (n) of these cells is illustrated. The second two inserts show cells in the body of the fan, (b) shows the clumps of polymorphonuclear leucocytes (P) always seen in these preparations and these cells often have the distorted nucleus typical of dead or dying cells. Insert (c) illustrates the formation of giant cells (G) seen in many of these cultures; this feature is typical of macrophage

FIGURE 1 GUINEA-PIG CELL MIGRATION

(A) Photomicrograph of the emigration of guinea-pig peritoneal exudate cells (PEC) Forty hours incubation. R: Red blood cells. C: Capillary tube.
a, b, c: Areas shown under higher magnification in Figure 2.
(x 16 magnification).

(B) Photomicrograph of the emigration of guinea-pig PEC derived from donor animals sensitised to tuberculin in the presence of 5.0 ug/ml soluble PPD. Forty hours culture. (x 16 magnification).

Cell cultures were fixed in methanol prior to staining with Geimsa


A
a
b
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R
C

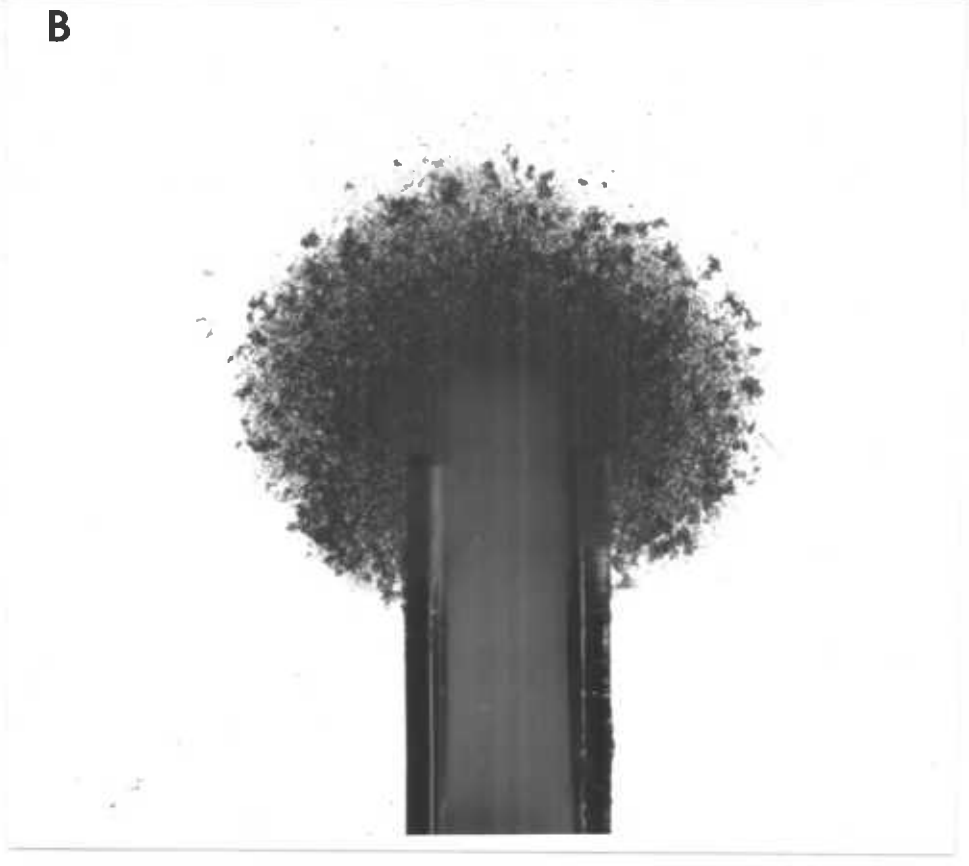

B

FIGURE 2 HIGH POWER PHOTOMICROGRAPHS OF GUINEA-PIG CELL MIGRATION (SEE FIGURE 1 (A))

a) Guinea-pig macrophages attached to the surface of the culture chamber beyond the leading edge of the cell fan (Figure 1 (A) a.). N: Kidney shaped nucleus typical of peritoneal macrophages. C: Foamy cytoplasm typical of attached, spread cells. (x 820 magnification).

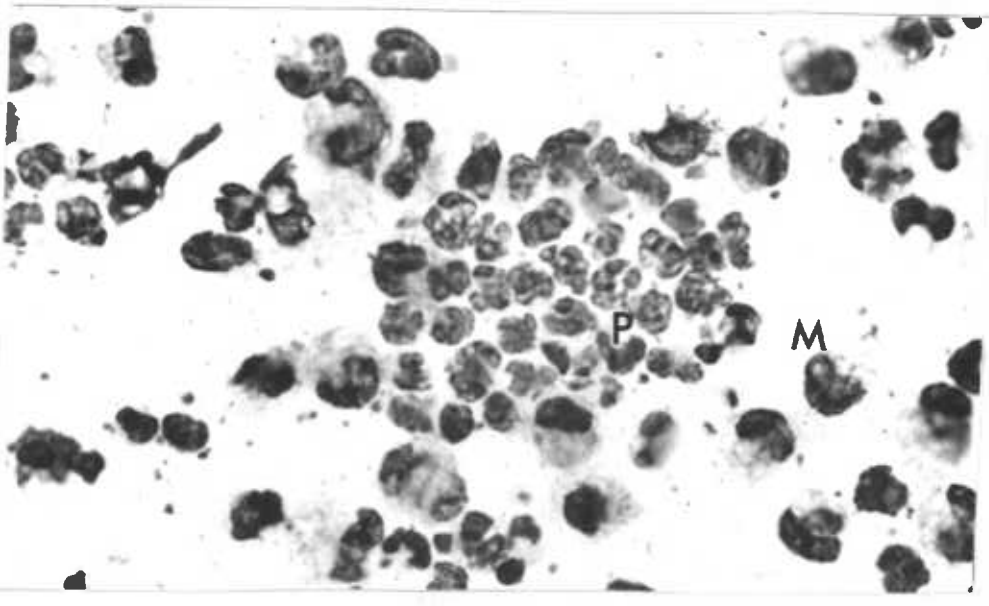
b) Migrating guinea-pig PEC (Figure 1 (A) b.). P: Polymorphonuclear leucocytes (PMNs). M: Macrophages. (x 820 magnification).

c) Migrating guinea-pig PEC (Figure 1 (A) c.). P: PMNs. M: Macrophages. G: Giant cell. (x 756 magnification).

Geimsa stain.

a

b



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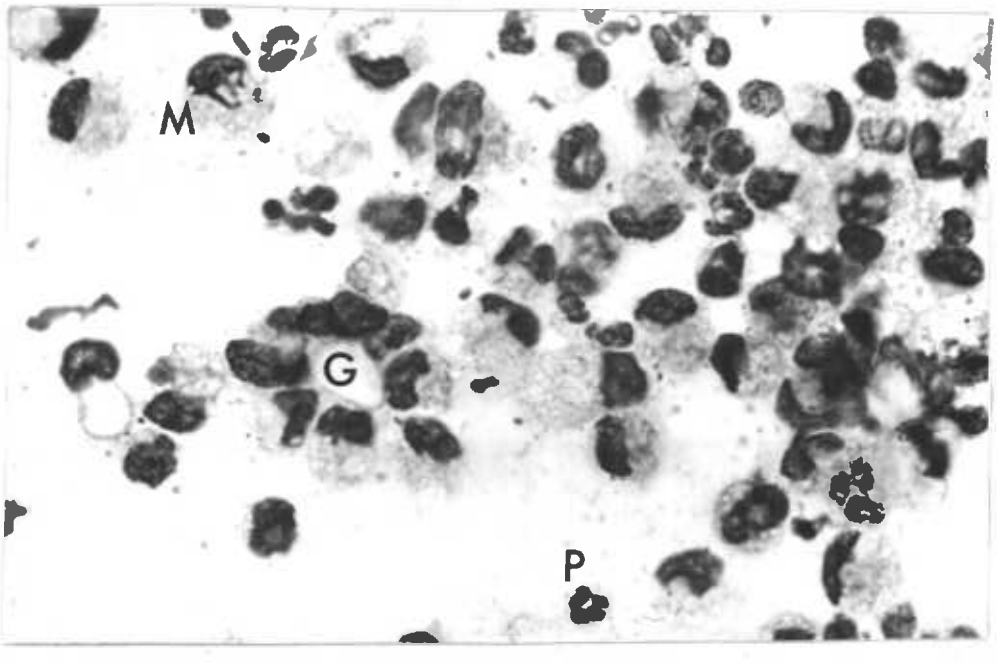


behaviour in long term cell culture (Allison and Davies, 1974). The cell distribution shown in the inserts occurs in both control (Fig.1A) and inhibited (Fig. 1B) cell migration and no differences in cell structure are visible in these light microscope studies.

Human peripheral blood leucocyte migration after 18 hours incubation is illustrated in Figure 3A and, by comparison, the migration of sensitised human leucocytes in the presence of specific antigen is shown in Figure 3B. The cell periphery is not as well defined in human cell migration as it is with most guinea-pig cell migrations (see Fig. 1). As with guinea-pig cell migration the pattern of cell distribution is similar between control and antigen inhibited cell migration and this observation is illustrated by Figure 4 inserts (a) and (b) which show the cell types and distribution in the migration illustrated in Figure 3A. Insert one (a) shows the cell distribution at the leading edge of the cell fan and a layer of small, heavily stained, polymorphonuclear leucocytes around the outer edge with some larger spread macrophage like cells. Insert (b) shows aggregations of polymorphonuclear leucocytes which parallels that seen in the guinea-pig cultures (see Fig. 2 insert (b)) however, the cell destruction (assessed visually by the shape and structure of the nucleus) does not appear as great as the guinea-pig PMN degredation (the human cell migration is for only 18 hours whereas the guinea-pig cells are cultured for 40 hours). The third insert (c) shows a high power view of the general cell distribution, showing small, dark staining lymphocytes (L), large mononuclear cells (M) and polymorphs (P).

The human peripheral blood cell population used in the migration assay contain a high percentage of contaminating red blood cells which appear after culture to form a network of channels through the fan radiating from the capillary tube. In guinea-pig peritoneal cell populations red cells make up 10-20% of the population and after migration they appear as an outer halo around the cell fan (Figure 1) and may lead to misleading measurements of the size of the white cell migration area.

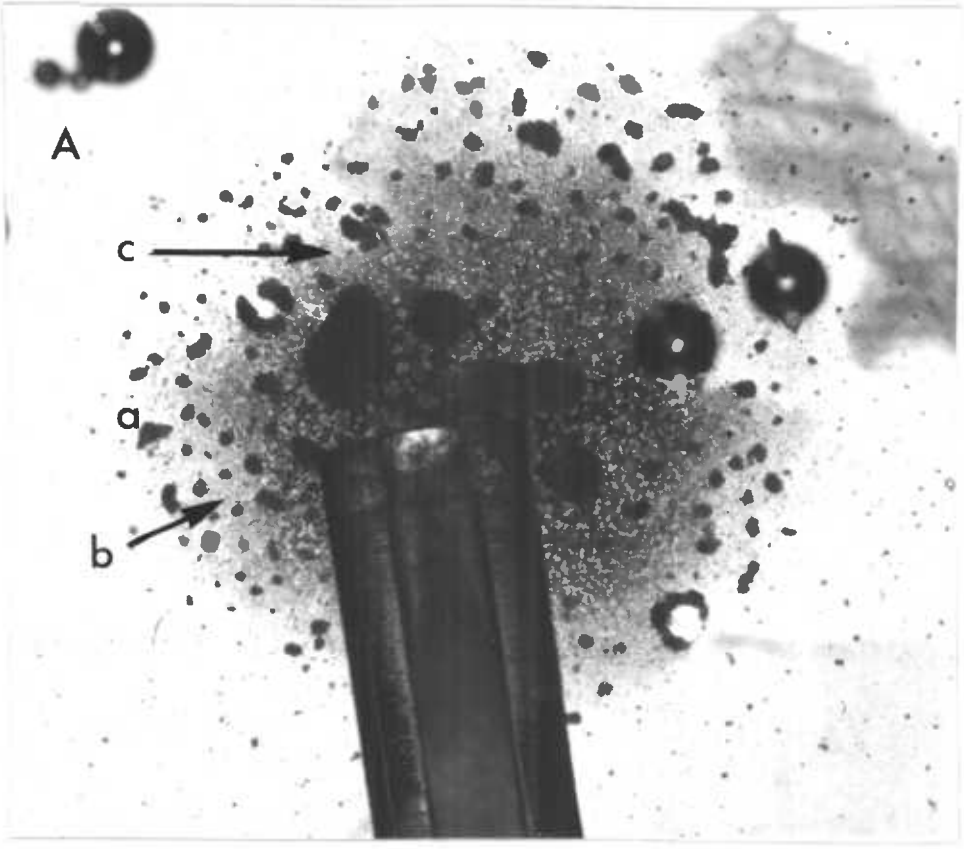
FIGURE 3 HUMAN PERIPHERAL BLOOD LEUCOCYTE MIGRATION

(A) Photomicrograph of the migration of human peripheral blood leucocytes (PBLs) in the absence of inhibitors. a, b, c: areas shown under higher magnification in Figure 4. (x 16 magnification).

(B) Photomicrograph of the emigration of PBLs from a donor sensitive to tuberculin in the presence of 20 ug/ml lyophilised Mycobaterium tuberculum (MTB). (x 16 magnification).

Geimsa stain.

000064



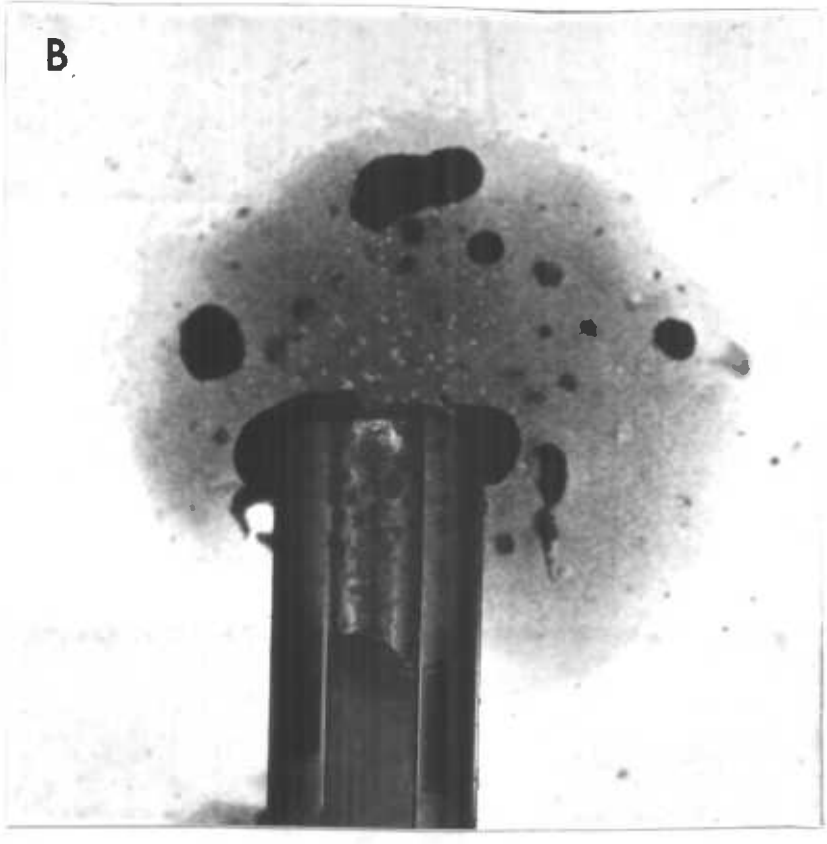


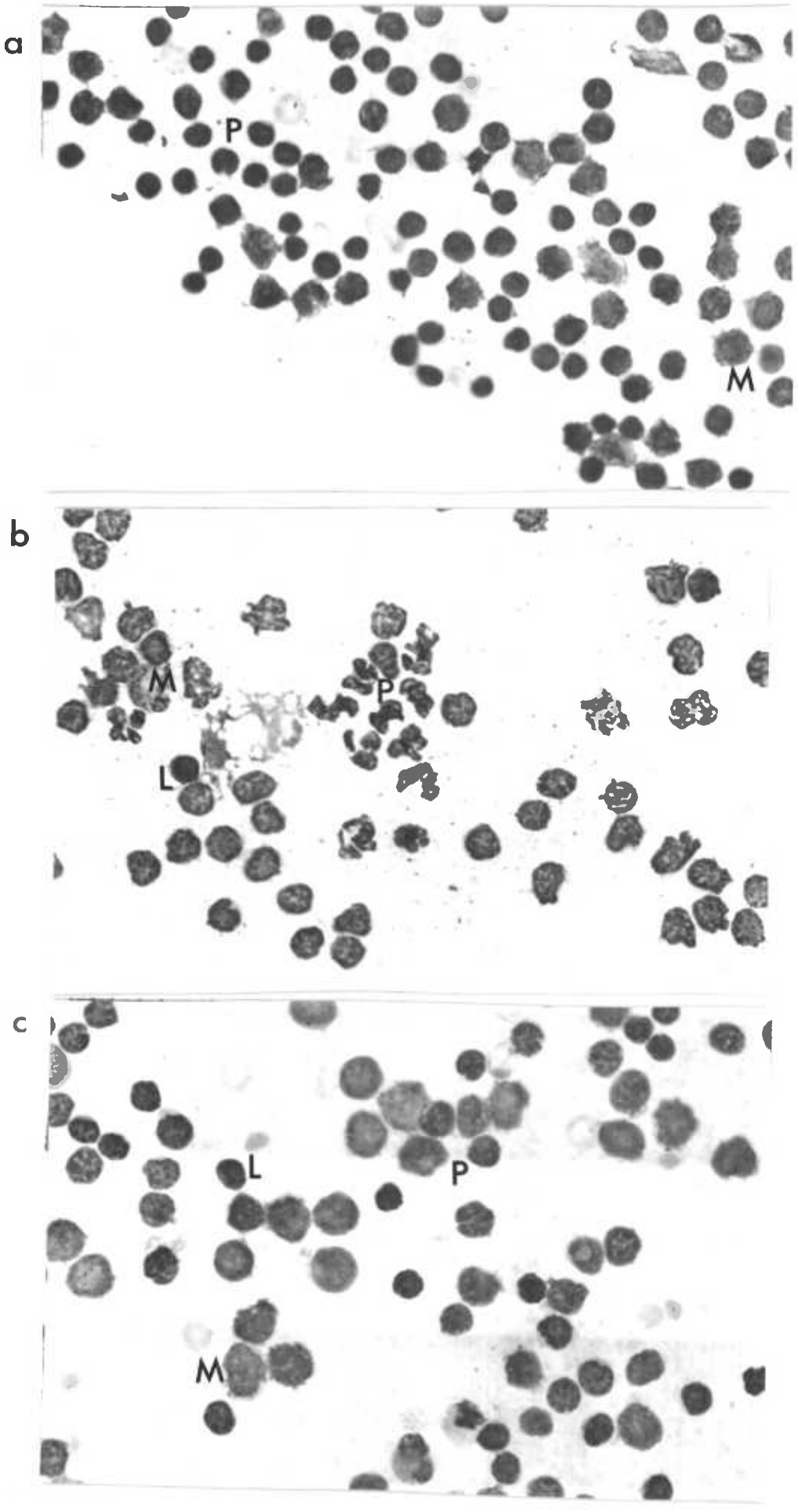
FIGURE 4 HIGH POWER PHOTOMICROGRAPHS OF HUMAN PBL MIGRATION (SEE FIGURE 3 (A))

a) View of the leading edge of the cell fan. P: PMN. M: Macrophages. (x 630 magnification).

b) High power view of the central area of the cell fan showing polymorph aggregates. P: PMNs. M: Macrophages. L: Lymphocytes. (x 820 magnification).

c) General high power view of the central area of the cell fan. P: PMNs. M: Macrophages. L: Lymphocytes. (x 820 magnification).

Geimsa stain.


a
P
M
b
M
P
L
c
L
P
M

b) THE ACTION OF INHIBITORY AGENTS ON CELL MIGRATION

The time course of cell emigration has been defined using time lapse cinematography in order to determine the optimum time for measuring the area of emigration. The migration of both guinea-pig PEC and human peripheral blood leucocytes in the absence of any inhibitors is shown in Figure 5. From this data it can be concluded that using guinea-pig cells the pattern of increase in migration area is sigmoidal with a linear increase in size from approximately 10 - 40 hours culture (B). Human leucocyte emigration (A) has a markedly different time course from guinea-pig PEC with an early burst of migration which slows considerably after 12 - 16 hours there being little further increase in the area of the cell fan up to 60 hours incubation.

The effects of several biological inhibitory agents on the time course of migration have been examined including antigens (BGG, PPD), mitogens (PHA (P), CON-A) and lymphokines. The response of PEC from tuberculin sensitive guinea-pigs to increasing doses of tuberculin PPD is illustrated in Figure 6. The specific antigen causes a marked dose-related inhibition of cell migration with maximal inhibition occuring with doses of 30ug/ml and above. No dose of antigen tested (up to 1mg/ml) was able to completely inhibit cell migration and the dose-response curves to both PPD and other antigens tested were relatively shallow.

The ID 50's (that is the dose of material causing 50% of the maximal inhibition for that agent) for another antigen (BGG), two mitogens (PHA and ConA), lymphokine and an inhibitory proteglycan isolated from guinea-pig Kurloff cells (Revell, Dean, Vernon-Roberts, Muir & Marshall, 1972) are compared with PPD in Table 3. PPD is the most active of the compounds measured in terms of ug protein/ml. However, the range of ID 50's for both antigens is wide compared with material directly affecting the macrophage (i.e. lymphokine) and this may be due to variable reactivity of the lymphocytes from individual animals even though immunised with the same dose of antigen. Similar variations in reactivity are also seen in skin reactions to antigen in both sensitised animals and man.

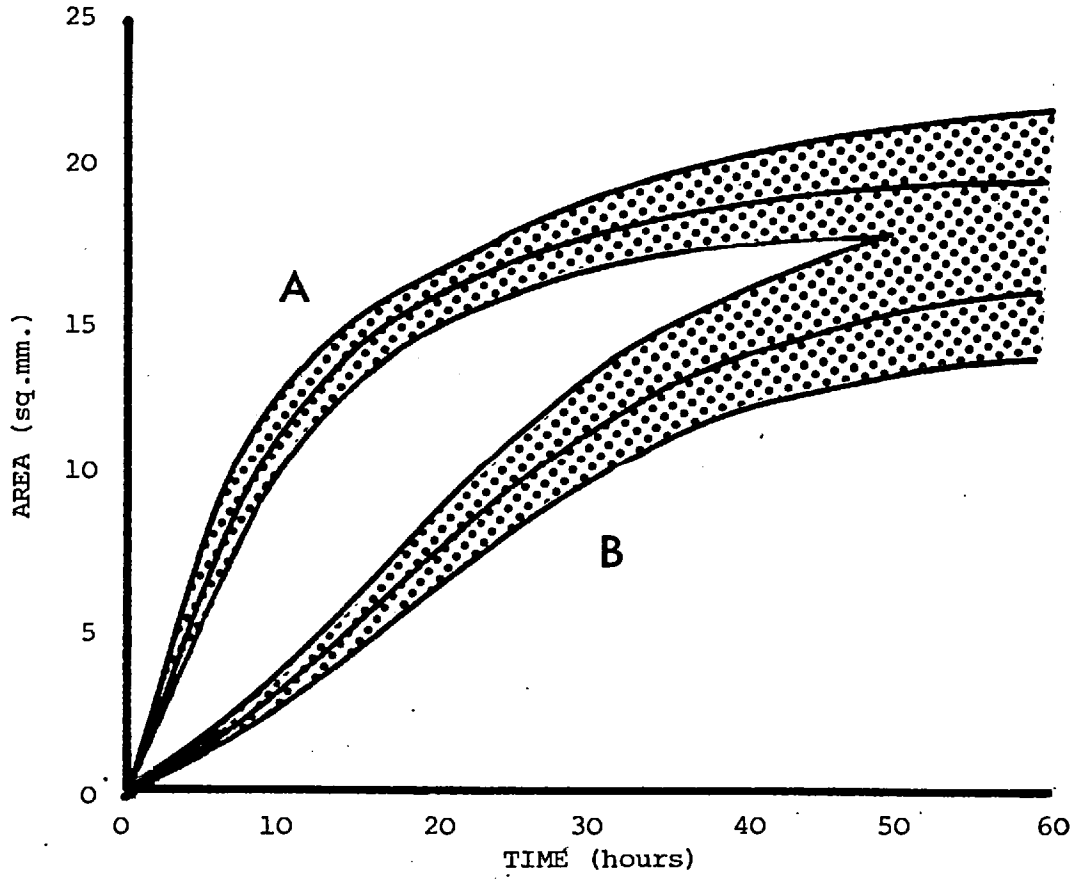


FIGURE 5 THE TIME COURSE OF GUINEA-PIG PEC AND HUMAN PBL CAPILLARY TUBE EMIGRATION

Each curve represents the mean ± standard deviation (SD) of eight cultures incubated for sixty hours in the absence of inhibitory materials.
All culture chambers contained Eagles MEM with 10% heat-inactivated FCS.
A Human PBL migration
B Guinea-pig PEC migration.

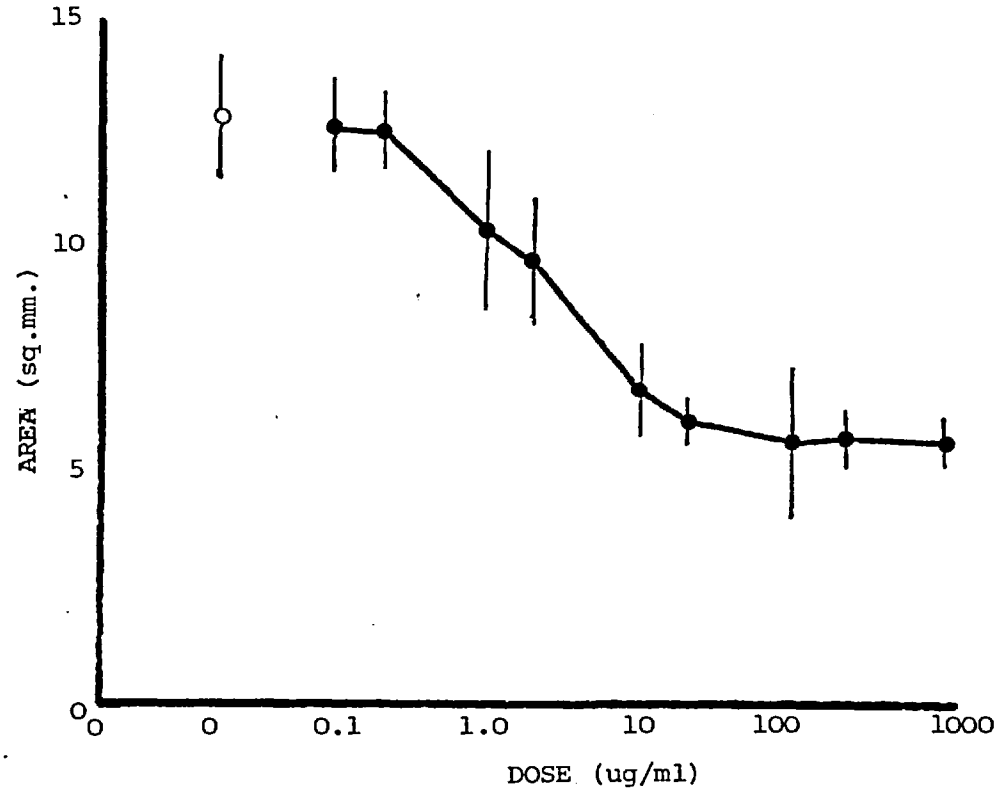


FIGURE 6 INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY SPECIFIC ANTIGEN

Donor cells from guinea-pigs sensitised to BCG in the presence or absence of increasing doses of tuberculin PPD. Number of replicate cultures (n) = 10. Mean $\pm$ SD. Cultures incubated for 40 hours. The dose of antigen causing 50% of maximal inhibition (ID_{50}) = 1.7 ug/ml.
o : Migration in the absence of antigen.
● : Migration in the presence of antigen.

TABLE 3 INHIBITION OF GUINEA-PIG CAPILLARY-TUBE MACROPHAGE MIGRATION

INHIBITORY MATERIAL	No. of Experiments (No. of Replicates)	MEAN ID_{50}* ± SD ug/ml	(RANGE) ug/ml
ANTIGENS			
PPD	10 (60)	1.93 ± 0.99	(0.16 - 3.90)
BGG	4 (28)	715 ± 255	(510 - 1150)
MITOGENS			
PHA	5 (25)	3.10 ± 0.80	(1.76 - 4.21)
CON-A	1 (8)	5.61 ± 1.63	(3.85 - 8.11)
KURLOFF PROTEOGLYCAN	2 (12)	14.1 ± 3.90	(10.2 - 18.0)
LYMPHOKINE	3 (18)	122.3 ± 26.4	(107 - 160)

* Inhibitory dose producing 50% of the maximum inhibition of cell migration obtained with each inhibitor

c) THE TIME COURSE OF CELL MIGRATION

It seemed possible that the time course of the inhibition of cell migration could prove useful in permitting discrimination between different inhibitory agents as well as providing an objective basis for determining the time of measurement of the responses. Additionally, such a recording system might provide a measure of the latent period between antigen stimulation of lymphocytes and the lymphokine action on the macrophage.

c. 1) EFFECTS OF ANTIGEN AND LYMPHOKINE ON THE TIME COURSE OF CELL MIGRATION

The time course of both guinea-pig PEC migration and human peripheral blood leucocyte migration were measured using time lapse cinematography (Methods vi.) c). Sensitised peritoneal exudate cells at a concentration of 4×10^6 cells/ml were migrated with increasing doses of antigen (PPD) in perspex 2 well migration plates for 65 hours. The migrations were filmed at 4 exposures/hour and the area of cell migration recorded (Figure 7). The results have been expressed as the difference in the area (sq.mm.) of cell fan containing antigen compared to the migration area of cultures in the absence of antigen. Thus difference = Control migration area - test migration area expressed as sq. mm. Doses of antigen up to 12.5 ug/ml were used and the degree of inhibition increased with time from approximately eight hours, indicating that, regardless of dose, 8 - 9 hours incubation was necessary before the inhibitory effect could be distinguished.

The effect of preformed lymphokine preparations on the time course of guinea-pig cell migration has also been examined. Three lymphokine preparations were tested and the results expressed as the difference between the control migrations and the migrations containing LK (control-test) expressed as sq. mm. (Figure 8). All three LK's tested caused migration inhibition after approximately two hours incubation and the inhibition seen increased up to at least 40 hours. After that time two LK preparations (B, C) showed some reduction in the difference and this implies that LK preparations may lose activity after prolonged culture (40 hours and longer). It was also apparent from these

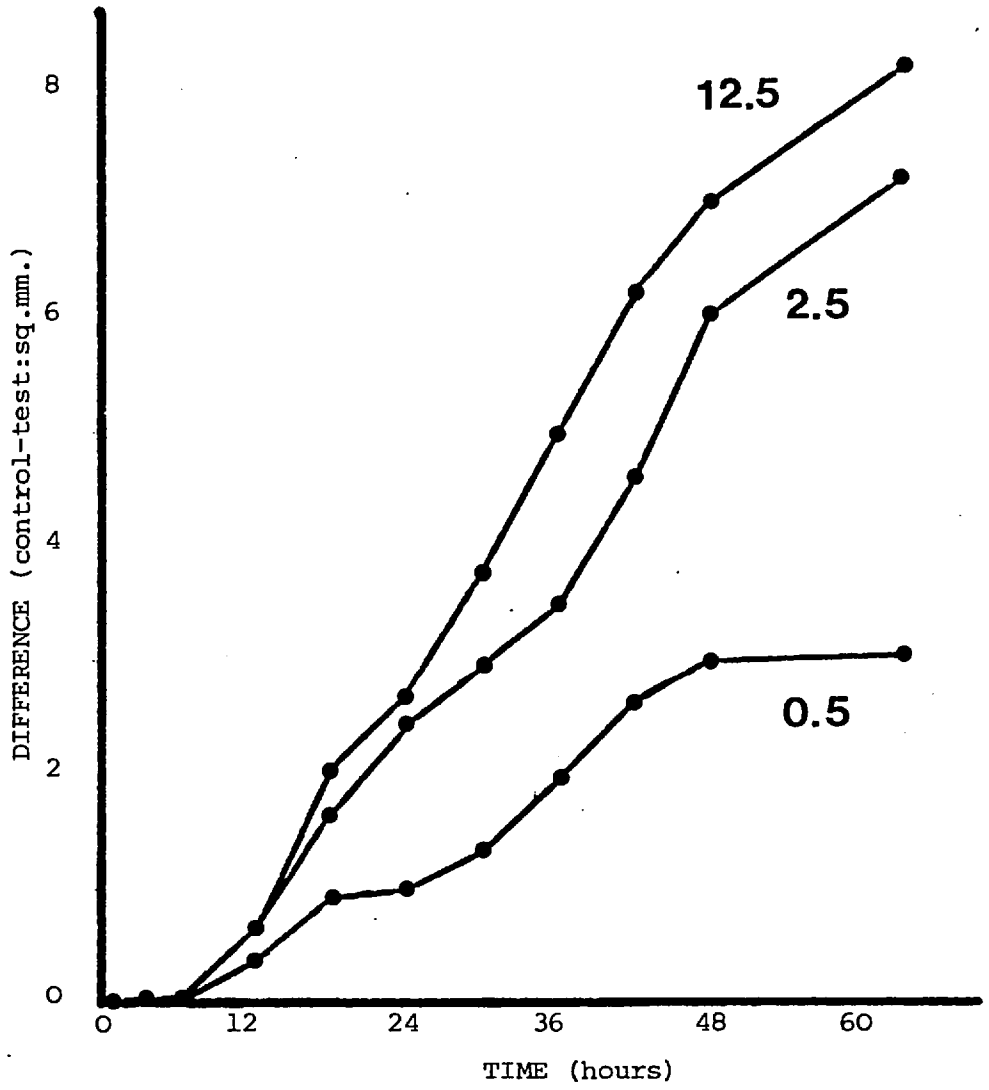


FIGURE 7 TIME COURSE OF INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY SPECIFIC ANTIGEN

Donor cells from guinea-pigs sensitised to BCG incubated in the presence of increasing doses of tuberculin PPD (ug/ml). Results expressed as "difference" (control-test) in sq.mm. of the area of migration. Cultures incubated for 65 hours. Each point represents the mean of three separate cultures.

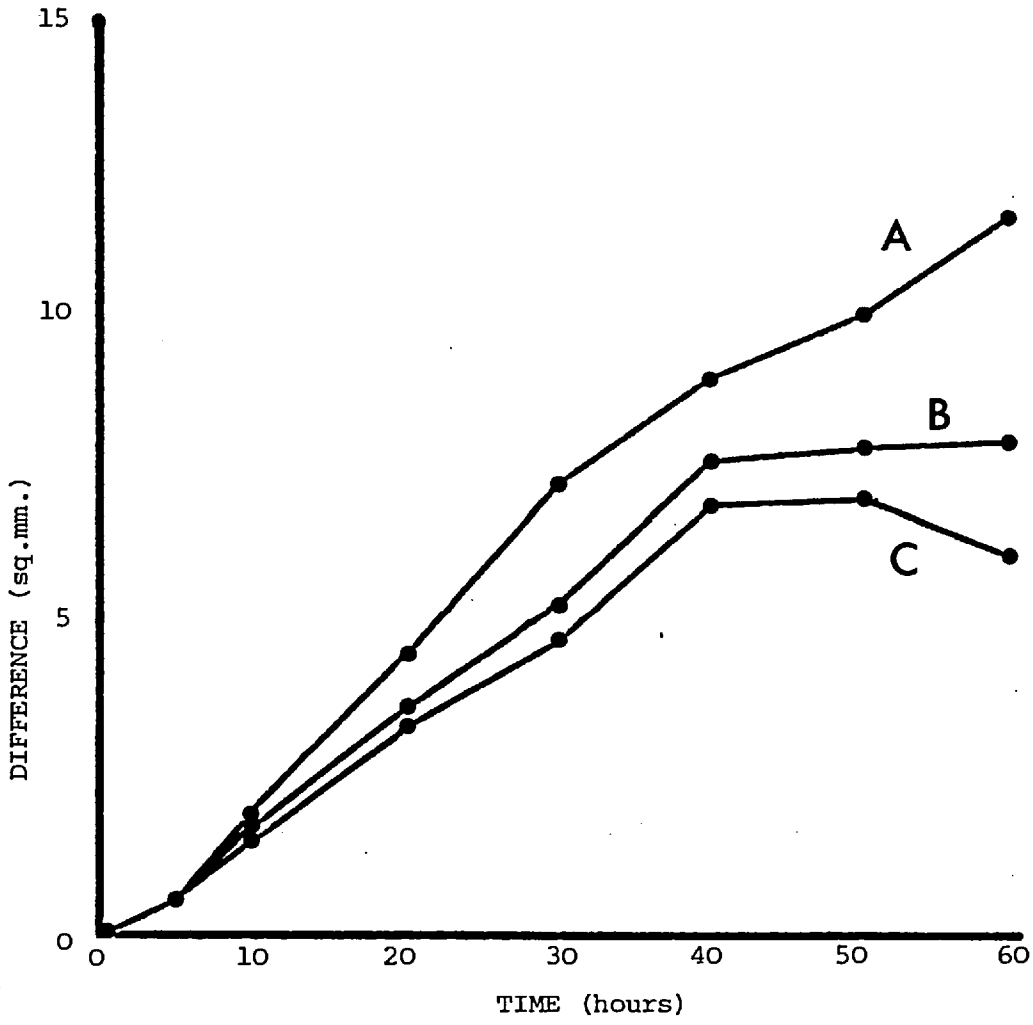


FIGURE 8 TIME COURSE OF INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY PREFORMED LYMPHOKINE

Donor cells from naive guinea-pigs incubated in the presence of ID_{50} doses of three preformed, AS purified lymphokine preparations (A-C). Results expressed as difference (control-test) in sq.mm. Cultures incubated to 60 hours. Each result represents the mean of three separate cultures.

preparations that measurement of the activity of LK's after approximately 40 hours may produce results inconsistent with the true potencies of the test materials.

The demonstration that LK preparations caused no visible inhibition in migration during the first two hours of culture was further investigated by incubating guinea-pig PEC with LK for four hours prior to culture (Figure 9). This treatment did not modify the two hour delay period observed if the LK's were added at the time of filming. The delay in inhibition when antigen was used (8 - 9 hours) was similarly investigated. Peritoneal exudate cells sensitised to tuberculin were incubated for 6 hours with 1.0ug/ml PPD and then placed in migration chambers and incubated in the presence of PPD (1.0 ug/ml). This pre-treatment caused a reduction in the time of onset of inhibition from 8 - 9 hours to 4 - 5 hours (Figure 10).

c. 2) EFFECTS OF MATERIALS WITH "MIF LIKE" ACTIVITY ON THE TIME COURSE OF CELL MIGRATION

Definition of the time course of migration inhibition may be of use in distinguishing qualitative differences between different inhibitory materials. The migration inhibition caused by two non-specific inhibitors is illustrated in Figure 11. The migration inhibition produced by a product of a virally transformed lymphocyte continuous cell line culture (ODLA) occurred immediately, whereas, in contrast, the inhibition to KPP (a proteoglycan derived from guinea-pig splenic,Kurloff cells) was similar to PPD, being delayed in onset for six to eight hours. This point is further illustrated in Figure 12 which shows the differences in onset of inhibition between the non-specific plant mitogen phytohaemagglutinin (PHA) and the specific protein antigen bovine gamma globulin (BGG) inhibiting the migration of cells from an animal previously immunised to the antigen. The relative inhibitory time courses of these two materials clearly differed during the first twelve hours of culture, there being a 6 - 8 hour delay in the onset of inhibition relative to controls in the cells responding to antigen in contrast to the immediate onset of inhibition during culture with PHA.

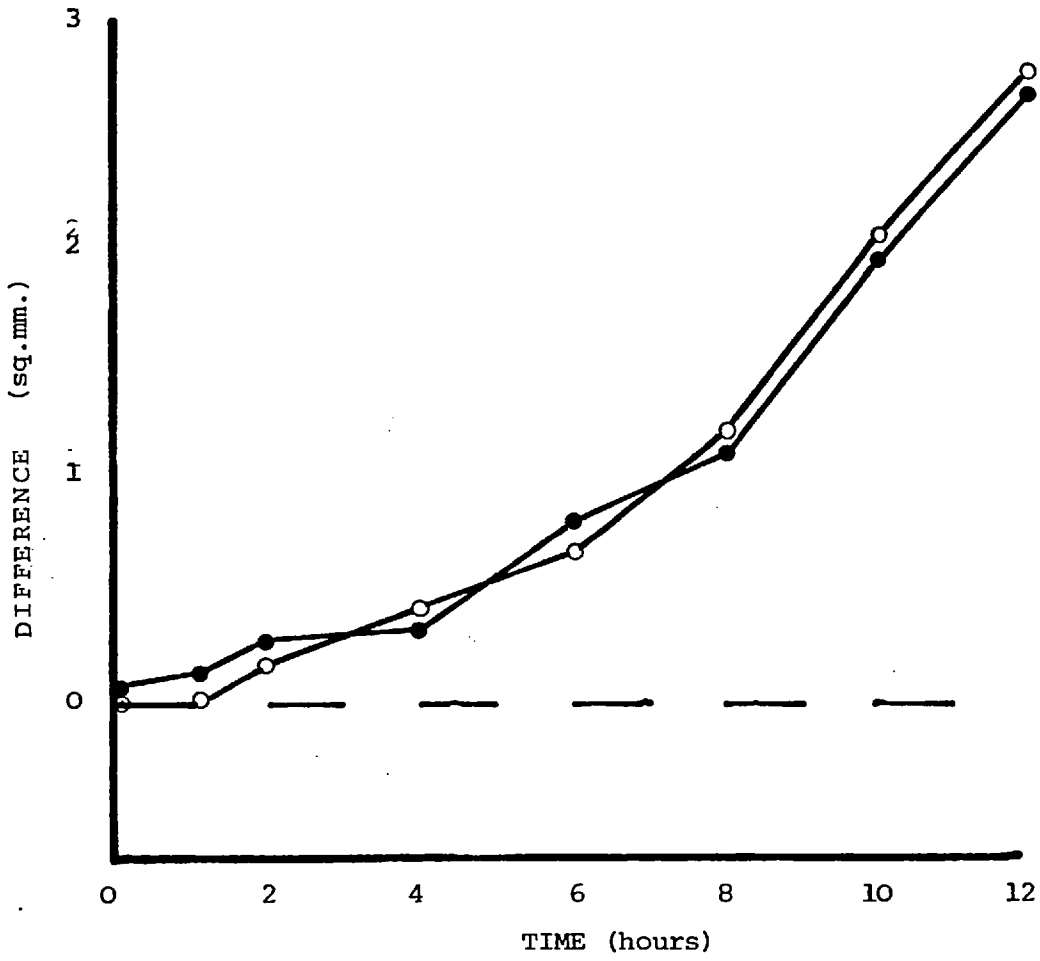


FIGURE 9 EFFECT OF PREINCUBATION ON THE TIME COURSE OF INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY PREFORMED LYMPHOKINE

Donor PEC from naive animals were incubated at 37°C in E-MEM plus serum with or without preformed lymphokine at the ID_{50} dose for 4 hours prior to being washed and used in the cell migration test. Migrations were then carried out in the presence of the same dose of LK as was used for preincubation. Results expressed as difference (C-T) in sq.mm.

o : Preincubation with no LK.

● : Preincubation with LK (ID_{50}).

The results following preincubation in the presence of antigen represent the means of duplicate cultures.

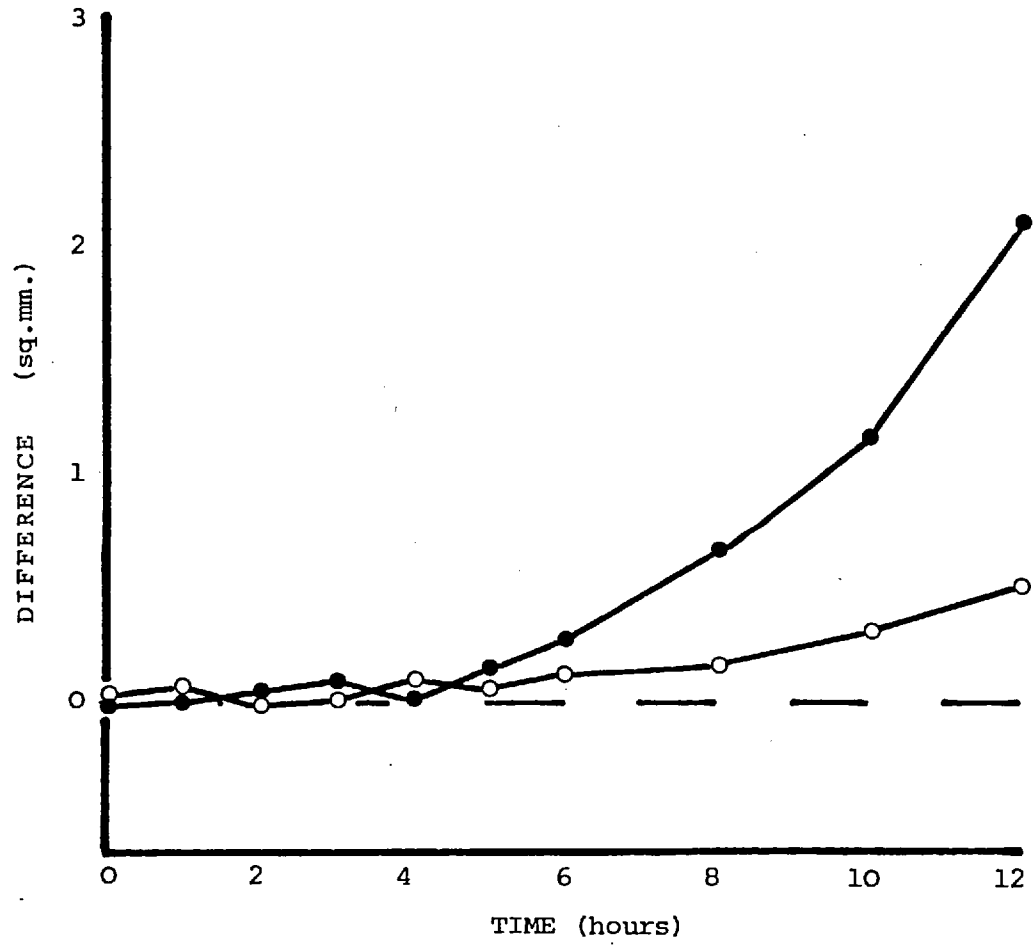


FIGURE 10 EFFECT OF PREINCUBATION ON THE TIME COURSE OF INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY SPECIFIC ANTIGEN

Donor cells derived from animals immunised to BCG. PEC were incubated for six hours at 37°C with or without tuberculin PPD (2.0 ug/ml = ID_{50}) prior to washing and packing into capillary tubes for migration. Cells were then migrated in the presence of 2.0 ug/ml PPD for 60 hours. Results expressed as difference (C-T) in sq.mm.

● : Preincubation without antigen

o : Preincubation with 2.0 ug/ml PPD

The results following preincubation with PPD represent the means of duplicate cultures.

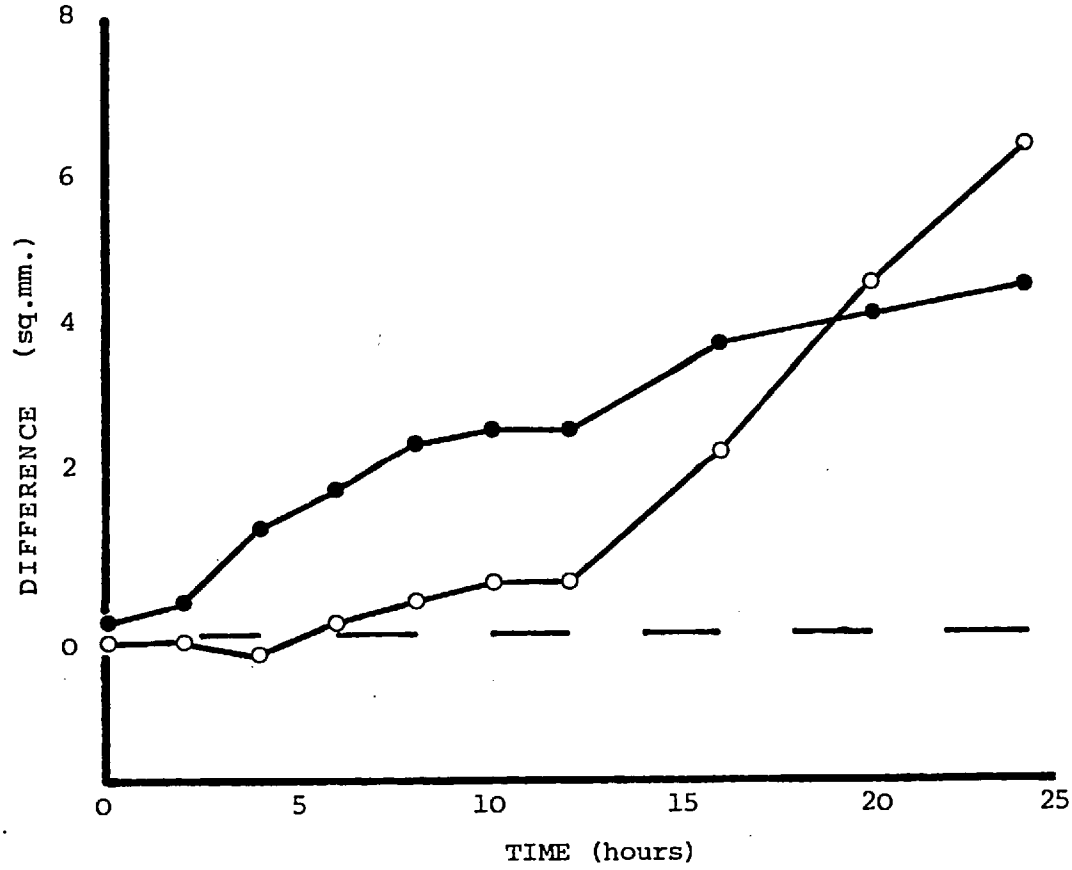


FIGURE 11 TIME COURSE OF INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY A CELL LINE PRODUCT AND A PRODUCT FROM GUINEA-PIG SPLENIC KURLOFF CELLS.

Donor cells derived from naive guinea-pigs. Migrations were carried out in the presence or absence of a 1:4 dilution (= ID_{50}) of a transformed lymphocyte cell line culture supernatant (ODLA) or 20.0 ug/ml (ID_{50}) of the Kurloff cell proteoglycan (KPP).
Cells were cultured for 60 hours. Results expressed as difference (control-test) in sq.mm.
● : ODLA 1:4 dilution
o : KPP 20.0 ug/ml
Each point represents the results of three replicate cultures.

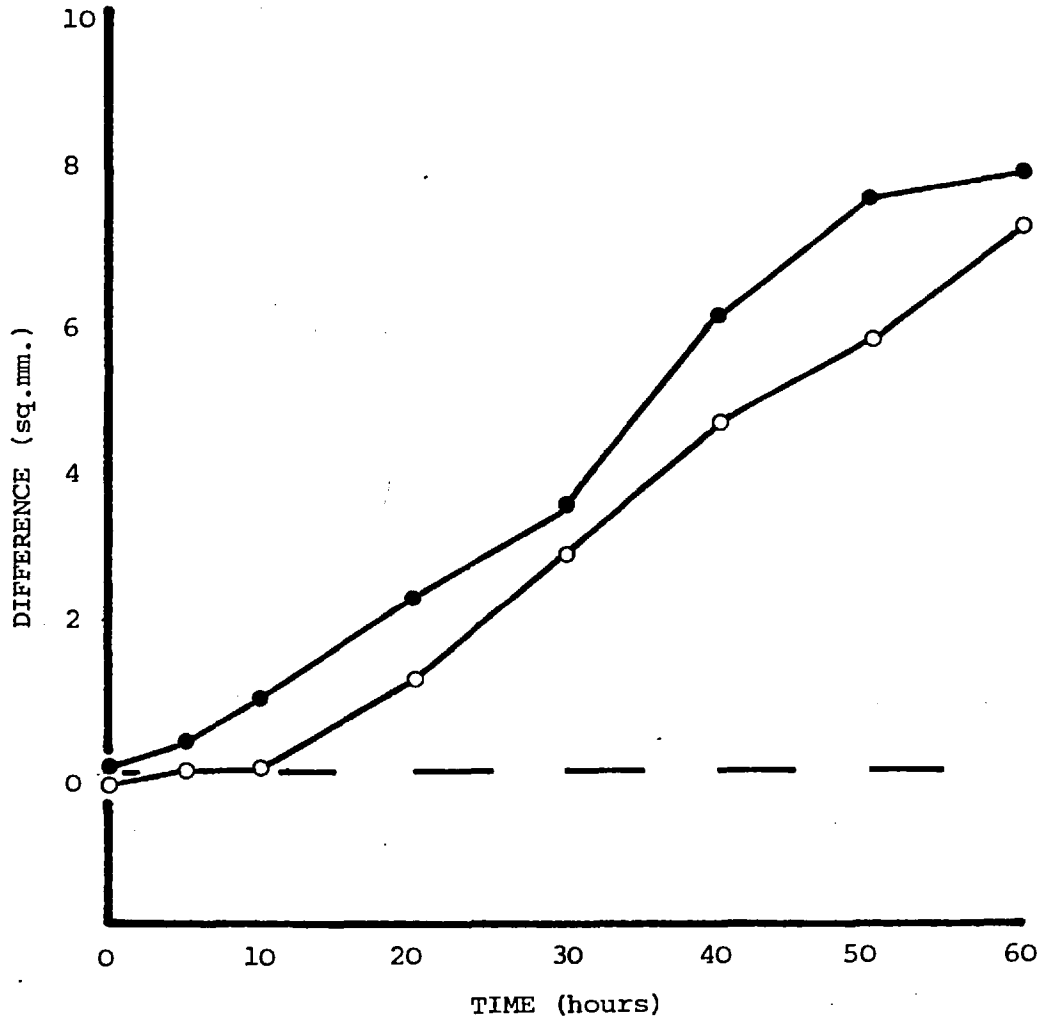


FIGURE 12 TIME COURSE OF INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY MITOGEN AND SPECIFIC ANTIGEN

Donor cells were derived from naive animals for migration with or without the plant mitogen PHA (3.0 ug/ml = ID_{50}) or from animals sensitised to the protein antigen BGG for migration using BGG as the stimulator of MIF generation (BGG at 750 ug/ml = ID_{50}). Cells were incubated for 60 hours in the presence of either PHA or specific antigen. Results expressed as difference (control-test) in sq.mm.

● : PHA 3.0 ug/ml

o : BGG 750 ug/ml

Each point represents the mean of duplicate cultures.

c. 3) OPTIMAL TIMES FOR MEASUREMENT OF CELL MIGRATION

Some indications of the optimum time for reading the results of migration inhibition experiments may be concluded from the time course data. From data presented in Figure 7 it can be seen that in the guinea-pig system the difference in the area of the cell fan between antigen treated cultures and control cultures increased for up to, at least, 60 hours. It would, therefore, be desirable to measure such cultures after such a time in culture in order to increase the significance of the differences in effect. Some guinea-pig lymphokine preparations in contrast whilst causing substantial migration inhibition during 24-28 hours in culture may lose their inhibitory action with longer periods of culture. This point is illustrated in Figure 8 where the difference between the area of control cell migration and the migration in the presence of LK preparation C diminished after 42 hours in culture.

In the case of antigen inhibition of human periphal blood leucocyte cultures the optimal time for reading the results in terms of maximum difference between untreated and treated cultures is restricted to a brief period between 16-24 hours (Figure 13). Inhibition appeared to be reduced after 24 hours and by 40 hours both test and control samples were the same size. This phenomenon has previously been reported (Brostoff, 1974) and is further illustrated in Figure 14 with the results expressed as "difference" between control and test. The peak of the difference between test and control cultures occurred after 18 hours and then fell rapidly. This "escape" from inhibition seen in human cell cultures using antigen as inhibitor was not seen when the plant mitogen PHA was used. In the latter situation the difference in migration area between the untreated controls and the PHA treated test cultures increased from 0 to 20 hours and remained constant up to at least 48 hours in culture (Figure 15). Such a difference between antigen and mitogen in the time course of cell migration inhibition casts some doubt on the validity of using PHA as a direct mimic of the action of antigen or lymphokine in human leucocyte migration cultures.

Finally, in this section the differences in the time course of cell migration between cells from different sources is illustrated. The

migration of peripheral blood leucocytes and peritoneal exudate cells both derived from guinea-pigs is shown in Figure 16. Peripheral blood leucocytes appeared to migrate at a faster rate than PEC and the cell fan had a larger area even after 60 hours of culture. These patterns of migration on the surface of the culture plate probably reflect the different cell types involved, however, further work would be needed to identify the causes of the differences.

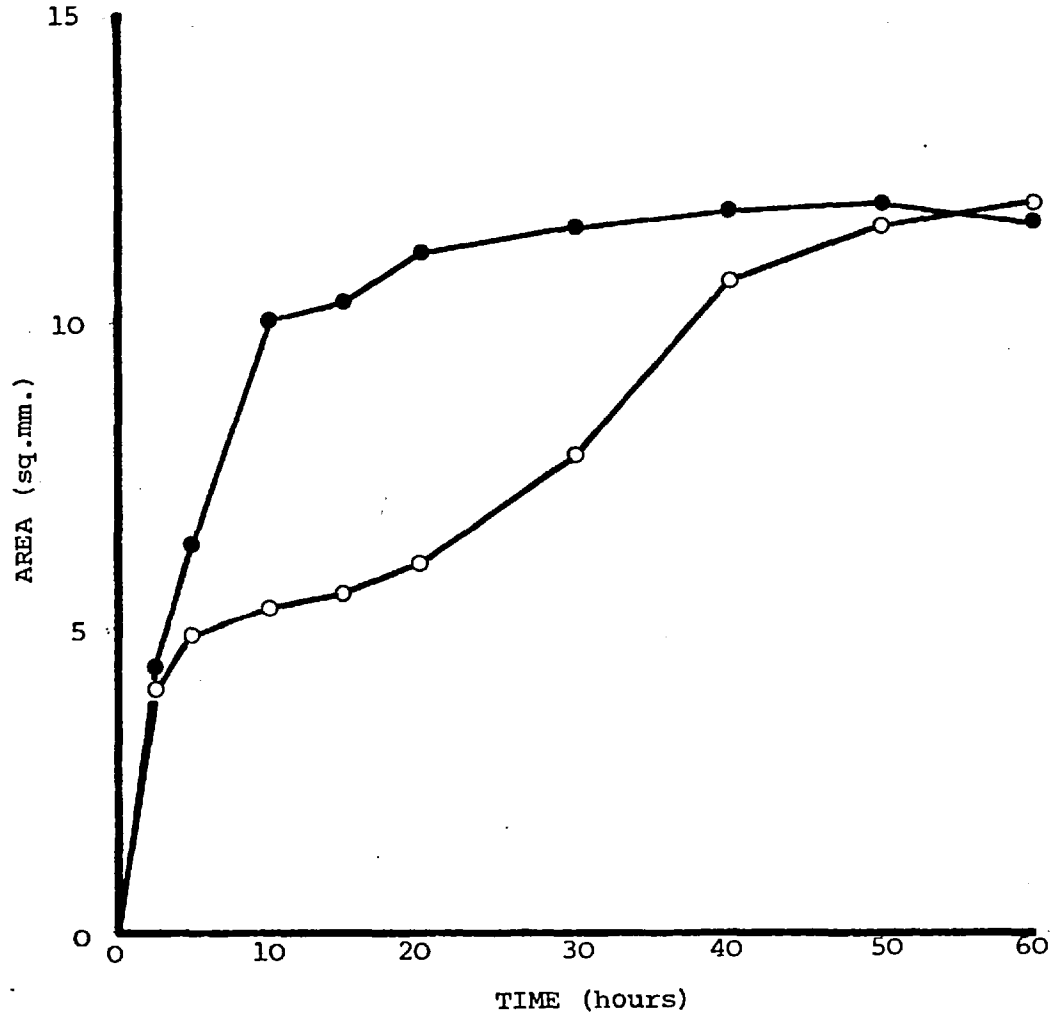


FIGURE 13 TIME COURSE OF INHIBITION OF HUMAN PERIPHERAL BLOOD LEUCOCYTES (PBL) CAPILLARY TUBE MIGRATION TO TUBERCULIN

Human peripheral blood leucocytes (PBL) from a donor sensitive to tuberculin were incubated in the presence or absence of freeze dried Mycobacterium (MTB: 50 ug/ml = ID_{50}). Results expressed as area in sq.mm.

● : No antigen

o : MTB 50 ug/ml

Each point represents the mean of duplicate cultures.

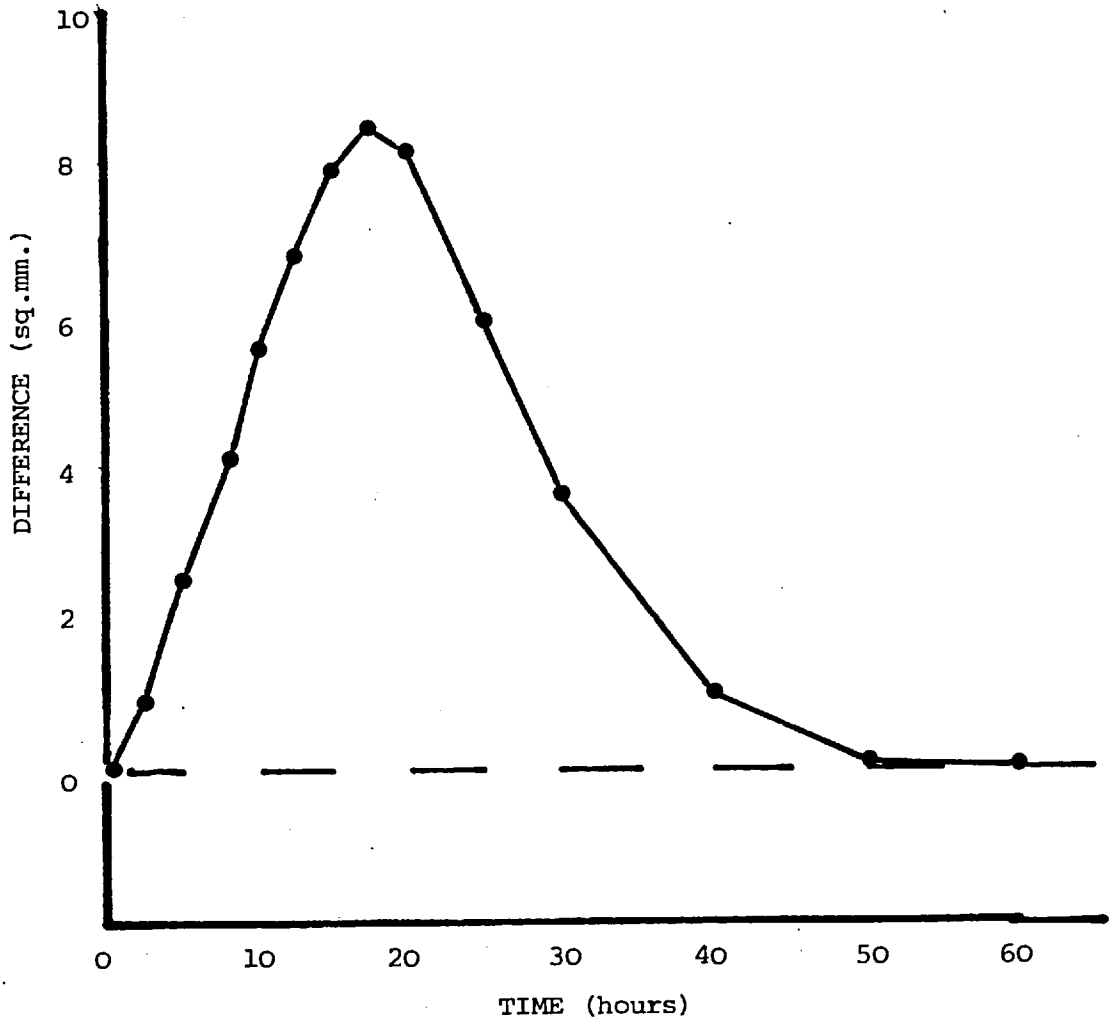


FIGURE 14 TIME COURSE OF INHIBITION OF HUMAN PBL CAPILLARY TUBE MIGRATION TO TUBERCULIN EXPRESSED AS DIFFERENCE BETWEEN CONTROL AND TEST CULTURES

Data taken from Figure 13. Results expressed as difference (control-test) in sq.mm.

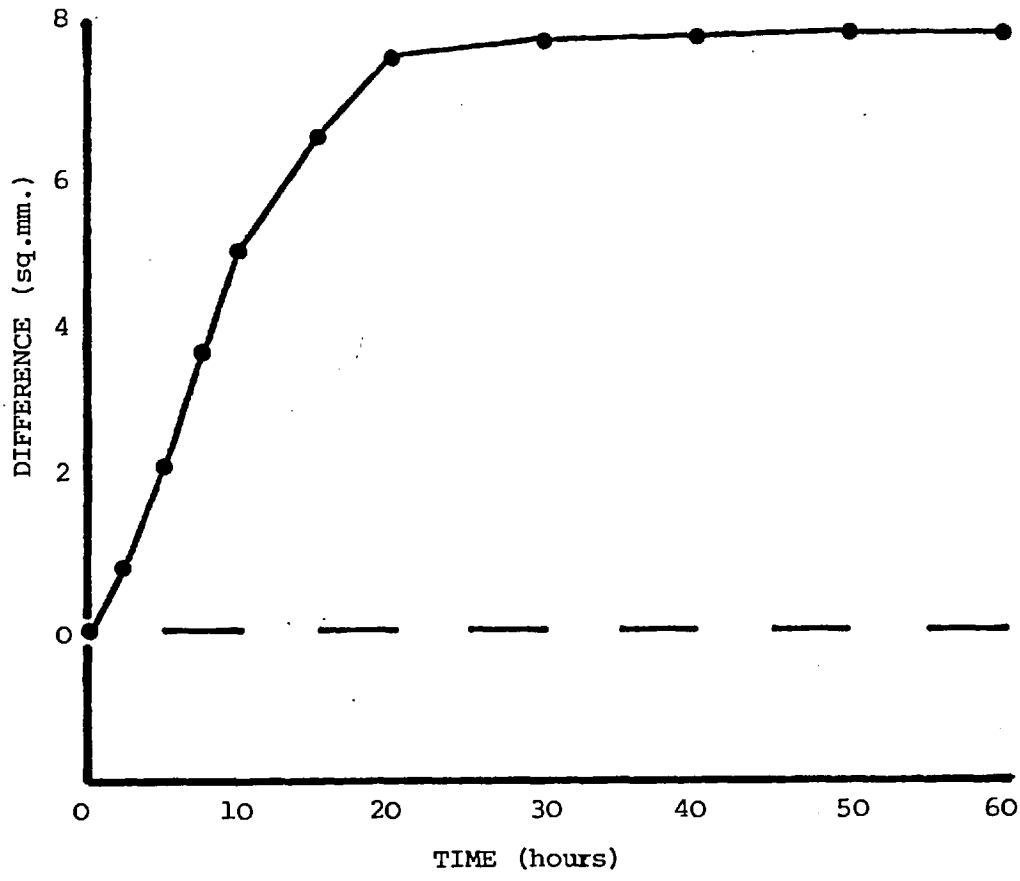


FIGURE 15 TIME COURSE OF INHIBITION OF HUMAN PBL CAPILLARY TUBE MIGRATION TO PHYTOHAEMAGGLUTININ (PHA)

Human PBL were incubated for 48 hours in the presence and absence of PHA (1.0 ug/ml = ID_{50} for inhibition of human PBL migration). Results expressed as difference (control-test) in sq.mm. Each point represents the mean of duplicate cultures.

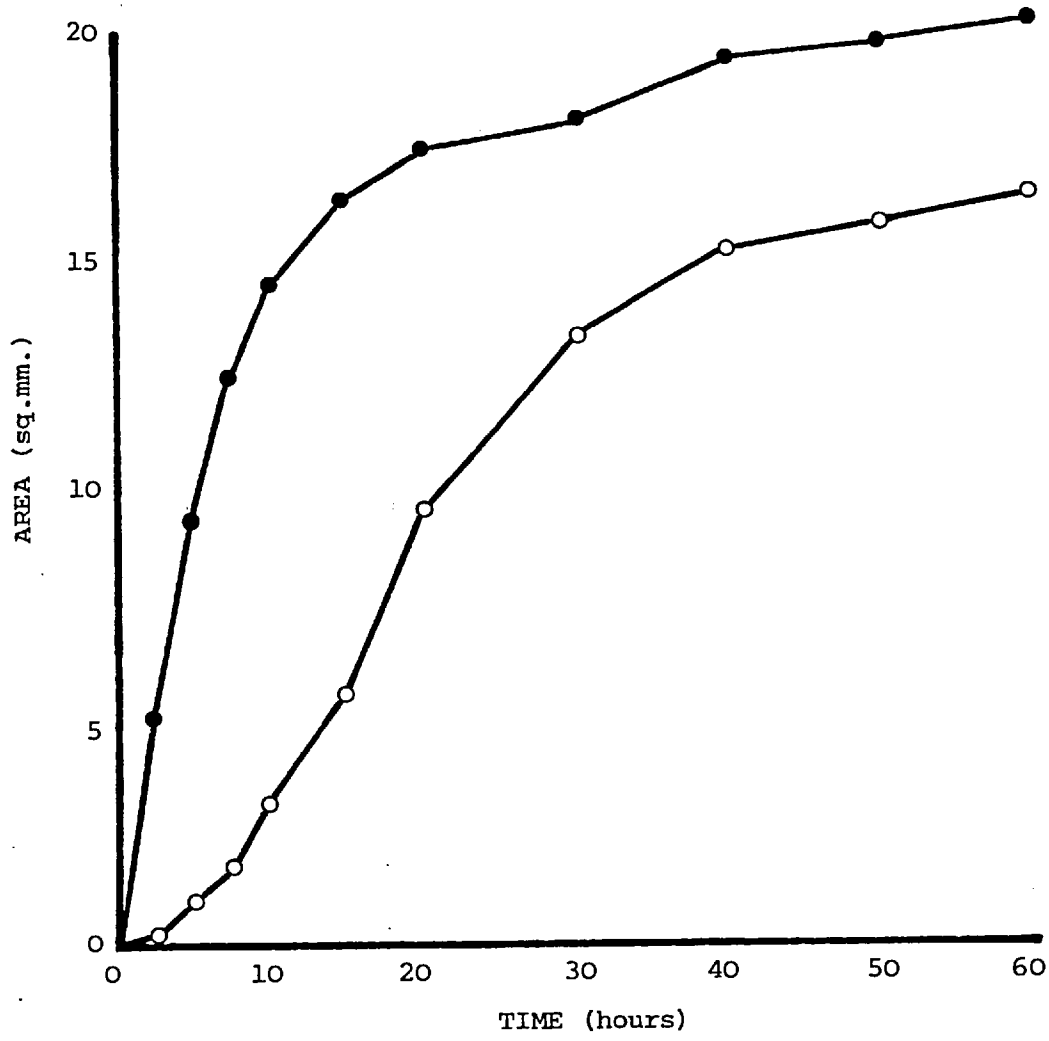


FIGURE 16 TIME COURSE OF CAPILLARY TUBE MIGRATION OF GUINEA-PIG PEC AND PBL

Both PEC and PBL cells were derived from the same donor animal. Cells were cultured for 60 hours in Eagles MEM containing 10% foetal calf serum.
● : Guinea-pig peritoneal blood leucocytes
o : Guinea-pig peritoneal exudate cells
Each point represents the mean of duplicate cultures.

3 ii) LYMPHOKINE HETEROGENEITY

In this section the guinea-pig macrophage migration inhibition assay has been used in multiple point bioassays to obtain quantitative comparisons between different lymphokine preparations. The potency ratios obtained using this procedure have been compared with those obtained for the same lymphokine preparations assayed independently in terms of their ability to stimulate lymphocyte transformation and to increase the vascular permeability in guinea-pig skin. Such a comparison has been used to assess the similarity of lymphokine preparations in terms of these three assays.

A series of seven lymphokine rich supernatants were prepared under closely similar laboratory conditions and partially purified by ammonium sulphate fractionation and extensive dialysis (see Section 2: Methods ix) and x)). One of these preparations was arbitrarily designated as the standard and the potencies of the remaining six preparations were assayed in terms of the standard.

a. STANDARDISATION OF REFERENCE LYMPHOKINE PREPARATION

Prior to assay of the six unknown preparations, aliquots of the standard lymphokine (LK-1) were assayed in a 3 + 3 parallel bioassay in order to define the dose response relationship and to examine the possible variation between sample aliquots.

The dose response curve for the designated standard lymphokine in terms of cell migration inhibition is shown in Figure 17. The dose range used for all the subsequent 3 + 3 bioassays of the standard was taken from the relatively linear portion of the curve (i.e. 25, 100 and 400 ug/ml of standard LK-1).

The three point dose response curves for three aliquots of the standard lymphokine assayed for guinea-pig cell migration inhibition activity are shown in Figure 18. Taking the potency ratio of one of the aliquots as 1.0 the remaining two aliquots had potencies of 1.09 and 0.89. Such a variation between aliquots of the standard was not considered too wide to preclude its use.

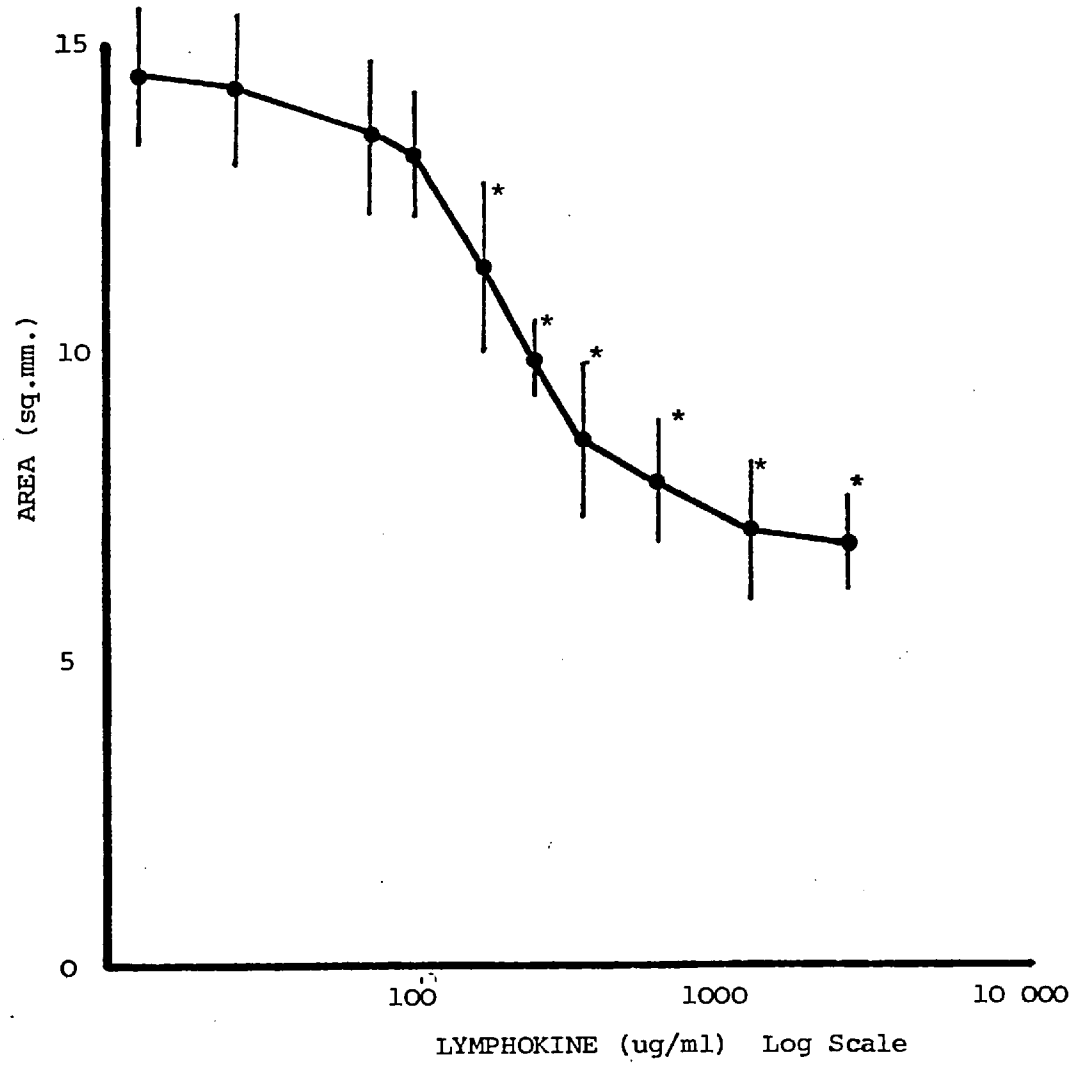


FIGURE 17 INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION IN THE PRESENCE IN INCREASING DOSES OF STANDARD LYMPHOKINE (LK-1)

Donor PEC were cultured in the presence of increasing doses of LK-1 (12.5 - 600 ug/ml freeze dried lymphokine).
*Significant reduction in migration. Students t-test, $p < 0.05$.
Each point represents the mean $\pm$ standard deviation ($n = 6$).
(Results expressed as area of migration in sq.mm).

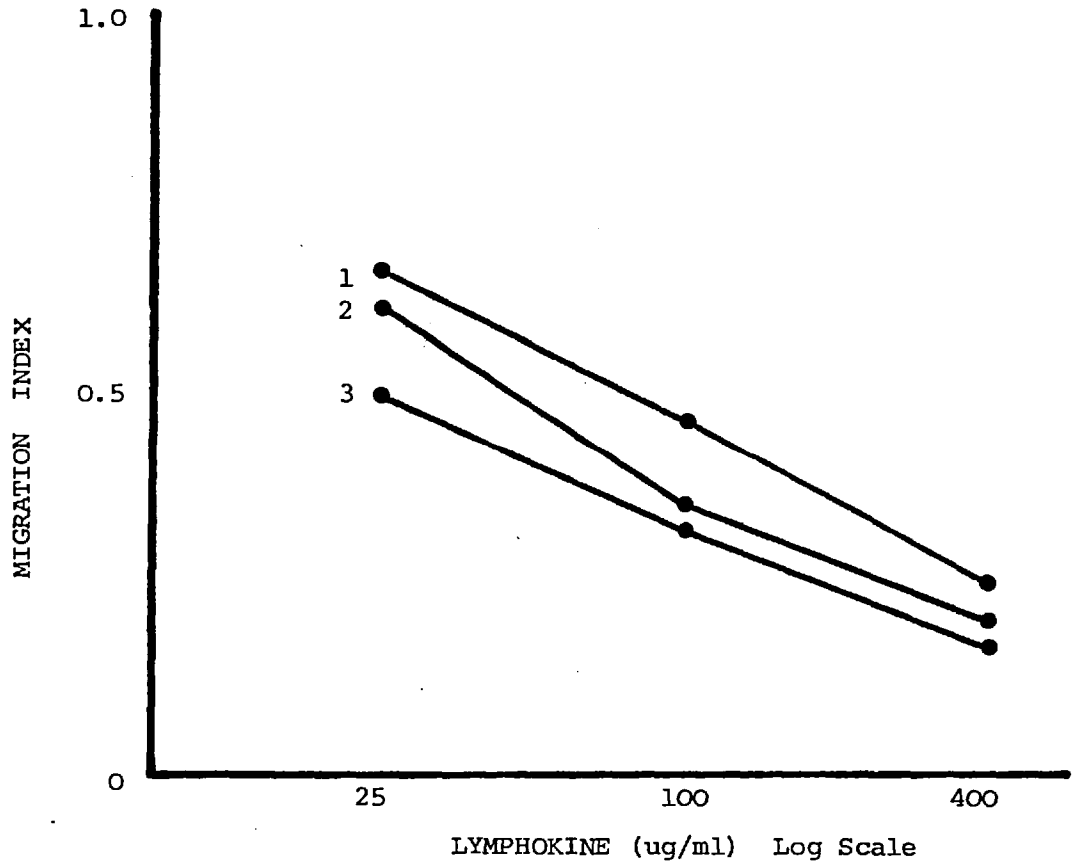


FIGURE 18 INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION IN THE PRESENCE OF ALIQUOTS OF LYMPHOKINE STANDARD (LK-1)

Donor PEC were cultured in the presence of increasing doses of three aliquots of LK-1 (1-3). Results expressed as a migration index

$$1 - \frac{\text{mean control} - \text{mean test}}{\text{mean control}}$$

See Section 2: Methods vi).a).

Number of replicate cultures (n) = 4

Potency ratio estimates (R) (with 95% confidence limits).

1 : 1.09 (0.96, 1.15)
2 : 1.00
3 : 0.89 (0.82, 0.97)

b. 3 + 3 ASSAY OF UNKNOWN LYMPHOKINES

1) CELL MIGRATION INHIBITION

Triplicate 3 + 3 bioassays of each of the unknown preparations measured against the standard LK were undertaken and an example of the dose-response curves obtained is shown in Figure 19. The unknown lymphokine (LK-5) exhibited less migration inhibitory activity than the standard over the dose range tested and the potency ratios for this preparation were 1.17, 1.35 and 1.73 in terms of the standard LK assigned the potency of 1.0.

The potency ratios and 95% fiducial limits for each of the unknown lymphokine preparations are listed in Table 4. Fiducial limits were calculated from Fiellers theorem (Finney, 1964; Colquhoun, 1972) and in the course of the calculations a full analysis of variance was carried out for each experiment. From Table 4 it can be seen that only one of the unknown LK preparations (LK-4) had a wide difference in potency ratio measurements and subsequent analysis of the variance of all the six unknown preparations showed that the wide scatter of potency ratios obtained with preparation LK-4 did not invalidate the overall assay of the six unknown LK's.

The calculations of the variance ratio (F) for linear regression, curvature, parallelism and linearity for each of the parallel line assays are listed in Table 5. Analysis of the data presented in Table 5 shows that differences between test and standard were consistently significant. However, there was also statistically significant ($p < 0.05$) deviations from parallelism between the standard and unknown preparations in 36% of the assays and the values for linear regression twice and curvature once varied significantly from normal. Whilst such values of F for parallelism, linear regression and curvature invalidate the potency ratio estimates for these assays, such variations have not been regarded by other workers as grounds for rejecting inclusion of the potency ratio estimates in the overall calculations (Humphrey, Lightbown, Mussett and Perry, 1953).

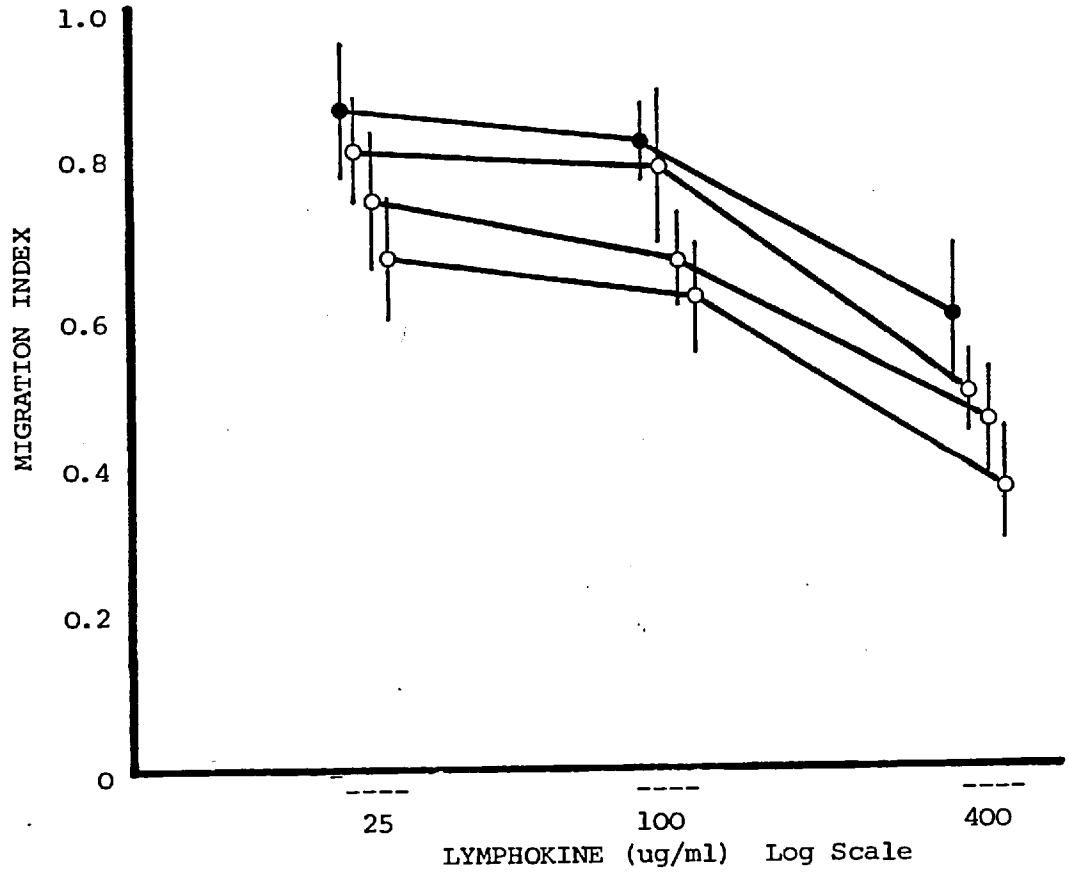


FIGURE 19 INHIBITION OF GUINEA-PIG PEC CAPILLARY TUBE MIGRATION BY THREE ALIQUOTS OF AN UNKNOWN LK (LK-5) ASSAYED AGAINST THE STANDARD PREPARATION

Donor PEC were cultured in the presence of increasing doses of unknown or standard lymphokine. Results were expressed as a migration index.

O : Each point represents the mean and standard deviation from six replicate cultures of three aliquots of unknown lymphokine assayed separately.

● : Each point represents the combined mean and standard deviation from three separate assays of standard lymphokine (n = 18, 6 replicates/assay).

TABLE 4 GUINEA-PIG PEC MIGRATION INHIBITORY ACTIVITY OF SIX GUINEA-PIG LYMPHOKINE PREPARATIONS ASSAYED AS A 3+3 BIOASSAY THREE TIMES WITH REFERENCE TO THE WORKING STANDARD (LK-1)*

Lymphokine preparation	Potency ratio determinations (with 95% fiducial limits) derived from three separate bioassays		
Standard (LK-1)	1.00	1.00	1.00
LK-2	1.00 (0.51-1.87)	1.26 (0.89-1.78)	1.49 (0.92-2.40)
LK-3	0.45 (0.17-0.98	0.49 (0.33-0.70)	0.58 0.28-1.10)
LK-4	1.05 (0.39-3.01)	0.24 (0.01-0.62)	0.58 (0.35-0.92)
LK-5	1.17 (0.40-2.89)	1.35 (0.63-2.75)	1.73 (1.03-2.89)
LK-6	1.91 (0.63-7.64)	1.73 (1.04-3.01)	1.93 (1.16-3.41)
LK-7	3.16 (1.96-5.33)	3.62 (2.54-5.32)	2.73 (1.85-4.12)

* Within each 3+3 bioassay n = 6
Each aliquot of unknown LK was assayed against a separate aliquot of standard

TABLE 5 CALCULATION OF THE VARIANCE RATIOS FROM 3 + 3 BIOASSAY OF SIX DIFFERENT LK PREPARATIONS ASSAYED THREE TIMES WITH REFERENCE TO THE STANDARD LK USING THE GUINEA-PIG PEC CAPILLARY TUBE MIGRATION INHIBITION TEST

SOURCE OF ERROR (expressed as variance ratios (F))

Lymphokine Preparation	Difference between standard & unknown	Linear Regression	Deviations from Parallelism	Linearity	Curvature
No.	Lp*	L_1	L_1'	L_2	L_2'
2	78.67	0.32	1.54	2.09	1.71
	136.96	0.10	0.04	0.78	1.21
	4.50	0.12	0.17	0.08	0.17
3	22.03	3.32	4.54	0.14	0.06
	169.70	2.1	19.49*	2.86	0.49
	45.40	2.88	4.53	0.003	0.71
4	22.93	0.22	0.16	0.32	0.004
	35.44	3.62	13.46*	0.49	2.54
	98.98	8.89*	1.18	0.51	3.68
5	17.7	0.58	0.66	0.24	0.001
	77.63	0.15	1.15	2.44	0.03
	38.63	0.003	0.48	0.003	1.25
6	60.28	0.15	4.42	3.12	0.38
	97.56	18.80*	9.35*	2.80	6.90*
	46.5	5.03	0.79	0.33	0.04
7	73.42	2.34	5.45*	2.32	0.97
	113.71	1.68	10.50*	1.94	0.02
	10.30	1.05	0.40	0.21	0.09

* Significant variation from normality ($p = 0.05$)

b. 2) 3 + 3 BIOASSAY OF LYMPHOKINES USING LYMPHOCYTE TRANSFORMATION AND INCREASED VASCULAR PERMEABILITY

Two other assays for lymphokine action have been used in conjunction with the macrophage migration inhibition test in order to measure the degree of heterogeneity between different lymphokine preparations. Firstly, the measurement of the uptake of tritiated thymidine by guinea-pig lymphocytes in response to the presence of lymphokines in culture and, secondly, the measurement, in vivo, of ^{125}I guinea-pig serum albumin extravasation in guinea-pig skin in response to intradermal injection of lymphokine. All the lymphokine preparations tested caused a dose-related increase in both tritiated thymidine uptake (lymphocyte transformation) and ^{125}I - GPSA accumulation (increased vascular permeability) and the mean potency ratios for the six preparations tested with the standard in these assays have been tabulated in Table 6. These results showed that there appeared to be considerable differences in the potency ratios obtained for a particular lymphokine preparation between the three assays. For instance, preparation seven (LK-7) had a mean potency ratio in the mitogenic assay of 1.61 compared with that for increased vascular permeability of 0.50 and for cell migration inhibition of 2.17. These results imply that there is a wide degree of heterogeneity of activity between ostensibly identical lymphokine preparations with respect, at least, to the three assays used.

c. ANALYSIS OF HETEROGENEITY

In order to evaluate statistically the differences between these lymphokines and to test whether the observed variations could be due to variations within the 3 + 3 bioassays or between the three methods used, an overall chi-squared (X^2) analysis of all the data from all three assay systems was undertaken. The method used was based on the X^2 tests for heterogeneity (Finney, 1964) as applied to the overall analysis of heterogeneity used to calculate the significance of differences between histamine antagonist preparations measured in a range of bioassay systems (Black et al., 1972). By using the overall analysis of X^2 these workers were able to demonstrate that the action of H_1 and non-H_1 groups of histamine antagonists were significantly different and that the differences

were not due to variations between methods of measurement. Demonstration of the heterogeneity of action of these compounds led to the definition of the H_2 histamine antagonists such as burimamide. Chi-squared analysis of the lymphokine data is presented in Table 7 with (a) the analysis within each assay, (b) the overall analysis of the preparation between the three assays and (c) the analysis of the differences between the methods. The P values obtained for the between preparation X^2 values in part (a) were highly significant for all three assays ($p<0.001$) whilst the X^2 values for the variations within the preparations measured 3 or 4 times using each assay were not statistically significant. These values demonstrate that the variation between one experiment and another when testing any preparation with any one of the three assay systems did not significantly contribute to the heterogeneity of the lymphokine preparations. Also that within each assay system there was a high degree of heterogeneity of potency between the seven LK preparations. Part (b) of Table 7 calculates the significance of the combined differences between methods in terms of the preparations and between the assays within each method. As expected from Part (a) the between assays within method X^2 was not significant, however, the between methods within preparations calculations were statistically significant ($p<0.001$) and so this figure must be taken into account in the calculation of the differences between preparations which, despite this inclusion remained statistically significant when calculated from the variance ratio ($p < 0.05$). Table 6 (c) further illustrates that despite the significance of differences between methods the variation between preparations remained considerable. Using this partitioning of the X^2 data the between methods p value was calculated from the variance ratio rather than the X^2 and was not significant whereas the between preparations value remained highly significant ($p < 0.001$) and the between test within preparation value as expected from (a) was not significant.

These results demonstrate that the three methods of assessment of lymphokine activity used have statistical validity and that there is a considerable degree of heterogeneity of activity between ostensibly similar lymphokine preparations measured using these three assays both within each assay system and between the three tests.

TABLE 6 MEAN POTENCY RATIOS OF THE LYMPHOKINE PREPARATIONS

Activity	Lymphokine preparations						
	Standard (LK-1)	LK-2	LK-3	LK-4	LK-5	LK-6	LK-7
Mitogenic	1.00	1.53	0.33	0.11	1.00	3.34	1.61
Inflammatory	1.00	1.38	0.88	1.79	0.70	1.35	0.50
Migration-inhibitory	1.00	1.25	0.51	0.62	1.42	1.86	2.17

Table derived from Bray et al, 1976

TABLE 7 POTENCY RATIO ANALYSIS (X^2) OF LYMPHOKINE ACTIVITIES BIO-ASSAYED WITH REFERENCE TO A STANDARD PREPARATION

Source of variation	Degrees of freedom	X^2	Mean Square	Variance Ratio	P
a) X^2 ANALYSIS (INDIVIDUAL ASSAY METHODS)					
Mitogenic assay:					
Between preparations	5	768.52	153.70	101.12	0.001
Between assays within preparations	18	27.43	1.52		NS
Total	23	795.95			
Inflammatory assay:					
Between preparations	5	54.57	10.91	20.98	0.001
Between assays within preparations	18	9.39	0.52		NS
Total	23	63.96			
Migration inhibition assay:					
Between preparations	5	76.15	15.23	35.42	0.001
Between assays within preparations	12	5.14	0.43		NS
Total	17	81.29			
b) OVERALL X^2 ANALYSIS (METHOD I)*					
Between preparations	5	548.16	109.63	3.60	0.05
Between methods within preparations	12	365.27	30.44		0.001
Between assays within methods	48	41.96	0.87		NS
Total	65	955.39			
c) OVERALL X^2 ANALYSIS (METHOD II)*					
Between methods	2	14.19	7.10	0.12	NS
Between preparations within methods	15	899.24	59.95		0.001
Between assays within preparations	48	41.96	0.87		NS
Total	65	955.39			

* Methods for the partitioning of X^2 further discussed in Bray et al., 1976

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3 iii) EFFECTS OF PHARMACOLOGICALLY ACTIVE MATERIALS ON CELL MEDIATED IMMUNE RESPONSES IN VITRO

a. HISTAMINE

Histamine has recently been implicated as a modulator of the activities of several inflammatory cell types and histamine receptors have been detected on the surface of some leucocytes (Melmon et al., 1972). Histamine in doses of 10^{-6} - 10^{-7}M has been shown to inhibit the release of IgE mediated antigenic histamine release from human peripheral blood leucocytes (Bourne, Lehrer, Cline & Melmon, 1971) and histamine and SRS-A release from monkey lung basophils (Ishizaka, Ishizaka, Orange & Austen, 1971). Antibody production may also be suppressed by histamine (Shearer, Melmon, Weinstein & Sela, 1972) and doses of histamine from 10^{-5} - 10^{-3}M (considerably higher than those used for the inhibition of histamine release) have been shown to suppress the lysis of DBA/2 mouse mastocytoma cells by specifically sensitised mouse splenic T-lymphocytes (Henney et al., 1972; Plaut et al., 1973). By employing the histamine antagonists diphenhydramine and burimamide (up to 10^{-4}M) these workers were able to demonstrate that the lymphocyte histamine receptor was of the H_2 type first postulated by Ash and Schildt (1966) and further delineated with the description of the specific H_2 antagonist burimamide (Black et al., 1972).

The possible involvement of histamine in the physiological control of T-cell function in terms of lymphokine secretion has been examined using the in vitro test of guinea-pig macrophage migration inhibition.

1) EFFECT OF HISTAMINE ON CELL MIGRATION

Histamine as histamine acid phosphate present throughout the culture at doses of 10^{-7} - 10^{-3}M histamine base was without effect on the migration of guinea-pig PEC during 40 hours in culture in the absence of antigen or lymphokine (Figure 20).

2) EFFECT OF HISTAMINE ON CELL MIGRATION INHIBITION

In the presence of 2.0ug/ml PPD tuberculin sensitised PEC were substantially inhibited from migrating. The addition of histamine (10^{-7} - 10^{-3}M) to the

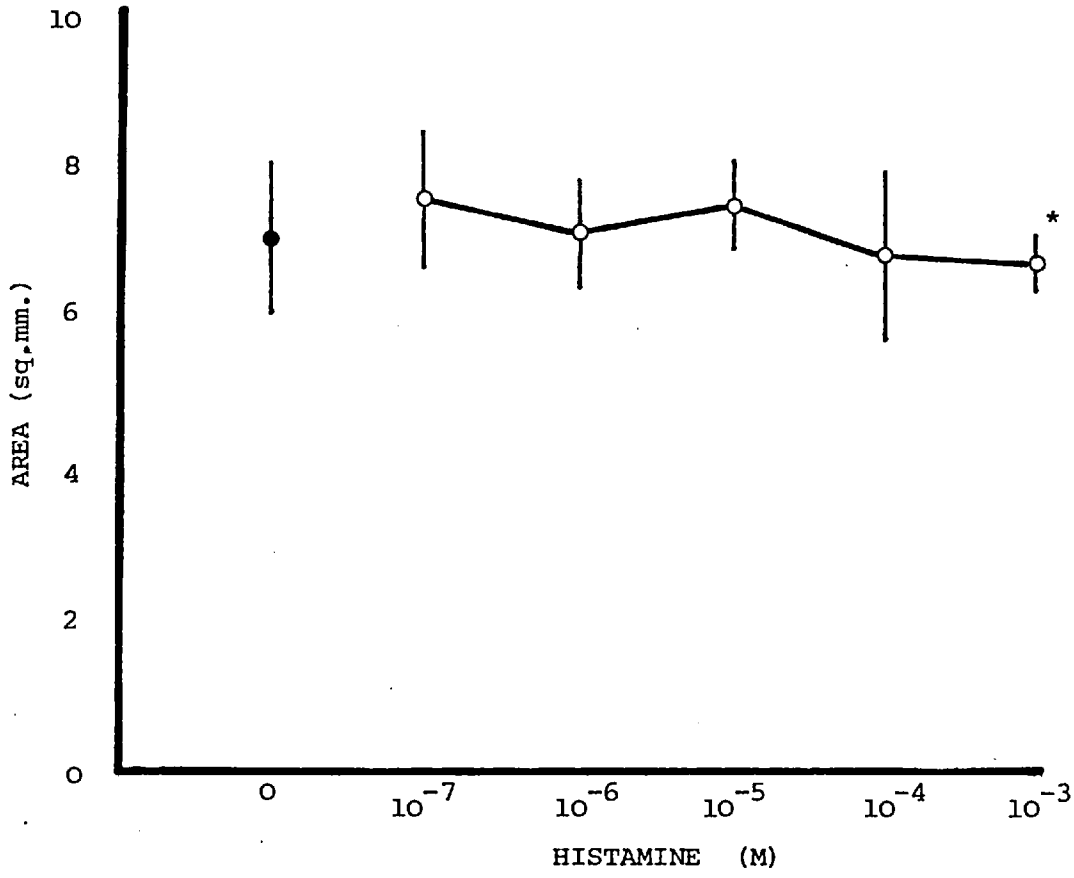

FIGURE 20 EFFECT OF INCREASING DOSES OF HISTAMINE ON THE EMIGRATION OF GUINEA-PIG PEC FROM CAPILLARY TUBES

PEC from unimmunised donor guinea-pigs were cultured for 40 hours in the presence of increasing doses of histamine acid phosphate (doses were calculated as for histamine base). Results expressed as area of migration in sq.mm. Each point represents the mean and standard deviation (SD) of eight replicate cultures.

● : Cell migration in the absence of histamine

o : Cell migration in the presence in increasing doses of histamine.

* No significant reduction in area. Students t test: $p > 0.1$.

culture medium throughout the culture period caused a slight though statistically significant (Students t-test:$p<0.01$) reduction in the inhibition with doses of 10^{-4} and 10^{-3}M (Figure 21). Such doses of histamine were considerably higher than those used to modify histamine release from basophils in response to IgE but were within the range of the doses shown to effect T-lymphocyte mediated lysis of target cells. Addition of an antihistaminase (semicarbazide) to the culture medium in order to inhibit any possible histaminase activity in the serum supplement had no significant enhancing effect on the action of histamine in the migration inhibition assay (results not shown).

3) EFFECTS OF HISTAMINE ON LK INDUCED MMI

From the above results it could be concluded that high doses of histamine were able to inhibit the migration inhibition caused by specific antigen but it was not possible to conclude whether the drug was affecting the lymphocyte or the macrophage response. In order to examine these alternatives the effect of histamine on the response of unsensitised PEC to preformed lymphokine was tested. A dose of 50ug/ml lymphokine caused substantial cell migration inhibition. The addition of doses of 10^{-7} - 10^{-3}M histamine to the culture medium for the duration of the culture period had no significant effect on the migration inhibition response to the lymphokine (Figure 22). The results of these experiments imply that the drug is likely to be affecting the response of the lymphocyte to antigen rather than the response of the target cell macrophages to lymphokine.

4) EFFECTS OF HISTAMINE ANTAGONISTS ON THE RESPONSES TO HISTAMINE IN MMI

In view of the description of H_2 receptor bearing T-lymphocytes being involved in cell mediated cytotoxicity (Henney et al., 1972; Plaut et al., 1973) attempts were made to inhibit the effect of histamine on antigen induced cell migration inhibition using histamine antagonists. The specific H_1 receptor antagonist mepyramine and the H_2 antagonists burimamide and metiamide were added to the cell migration culture medium throughout the assay. Doses of mepyramine maleate from 10^{-5} - 10^{-2} mg/ml failed to affect the response of BCG sensitised guinea-pig PEC to 2.0 ug/ml

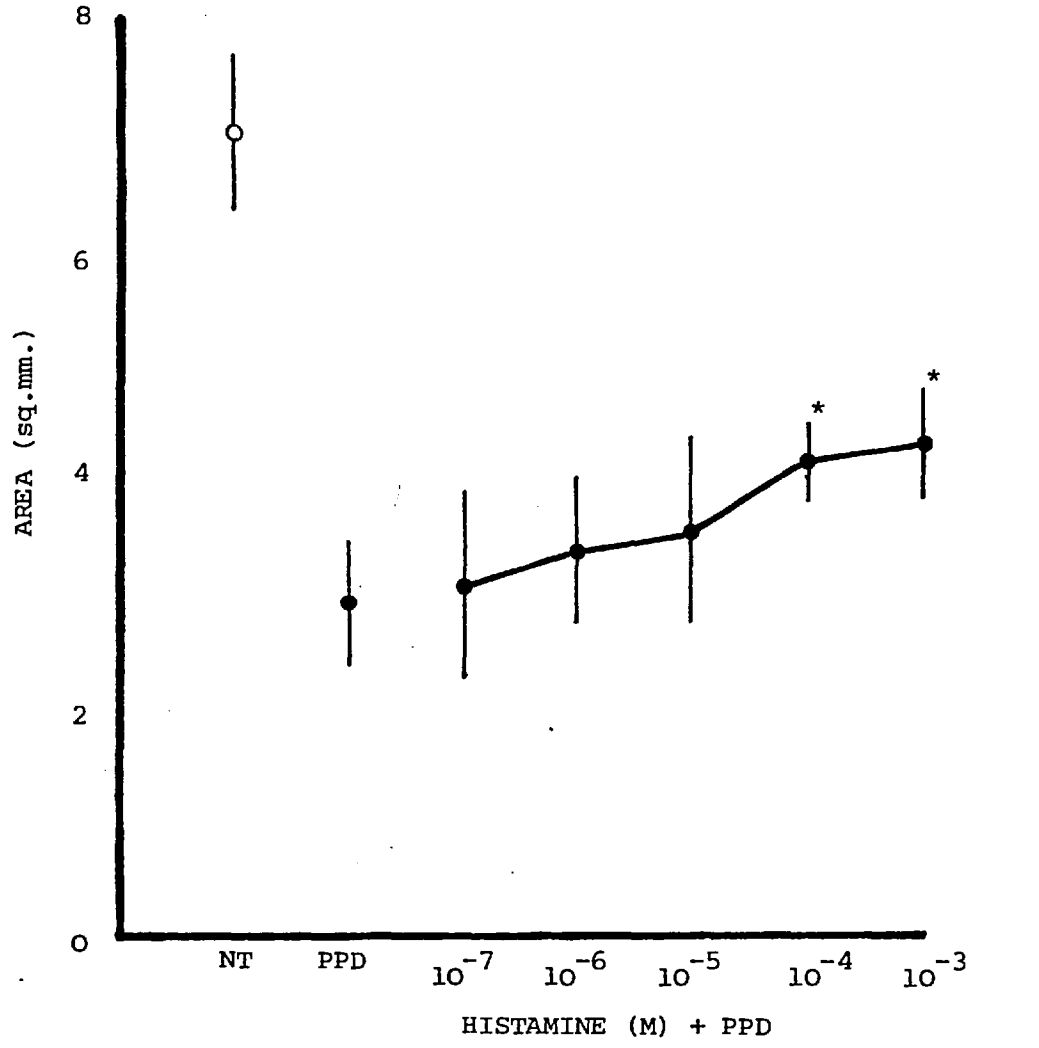

FIGURE 21 EFFECT OF INCREASING DOSES OF HISTAMINE ON THE CELL MIGRATION INHIBITION RESPONSE TO PPD

PEC from BCG immunised donor guinea-pigs were cultured for 40 hours in the presence or absence of 2.0 ug/ml PPD and increasing doses of histamine acid phosphate. Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.

o : Cell migration in the absence of antigen or histamine (NT)

● : Cell migration in the presence of 2.0 ug/ml PPD $\pm$ increasing doses of histamine.

* Applying the Students t test to the data the cell migrations in the presence of 10^{-4} and 10^{-3}M histamine were found to differ significantly from the cells cultured with antigen alone ($p < 0.001$).

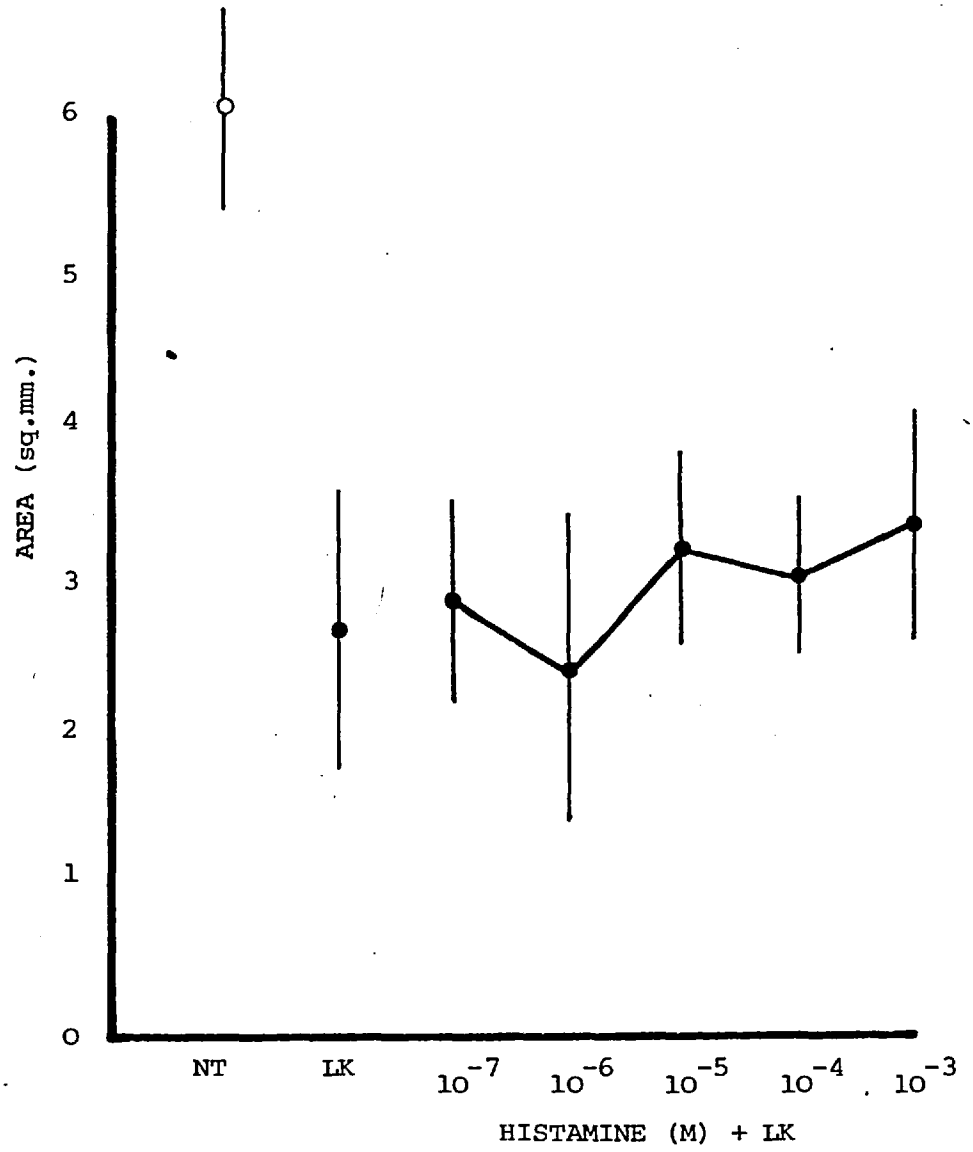

FIGURE 22 EFFECT OF INCREASING DOSES OF HISTAMINE ON THE CELL MIGRATION INHIBITION RESPONSE TO PREFORMED LYMPHOKINE

PEC from donor animals were cultured for 40 hours in the presence or absence of 50 ug/ml lymphokine (LK) and increasing doses of histamine acid phosphate. Results expressed as area in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.
o : Cell migration in the absence of LK or histamine (NT)
● : Cell migration in the presence of LK$^{+}_{-}$ histamine.

PPD. The drug was also without effect on the ability of 10^{-3}M histamine to inhibit the response to the same dose of antigen (Figure 23). Burimamide and metiamide at doses subsequently shown to inhibit histamine modification of lymphocyte DNA synthesis (Beets and Dale, 1979), (10^{-8} - 10^{-4}M) were added to the cultures in order to determine whether the response to histamine acted via H_2 receptors. Neither of the two drugs tested were able to effect the response to 2.0 ug/ml PPD nor were they able to suppress the effect of 10^{-3}M histamine on the antigen induced migration inhibition (Figure 24).

These experiments have not been able to indicate whether the effect of histamine on the lymphocyte response to antigen acts via a specific H_1 or H_2 receptor.

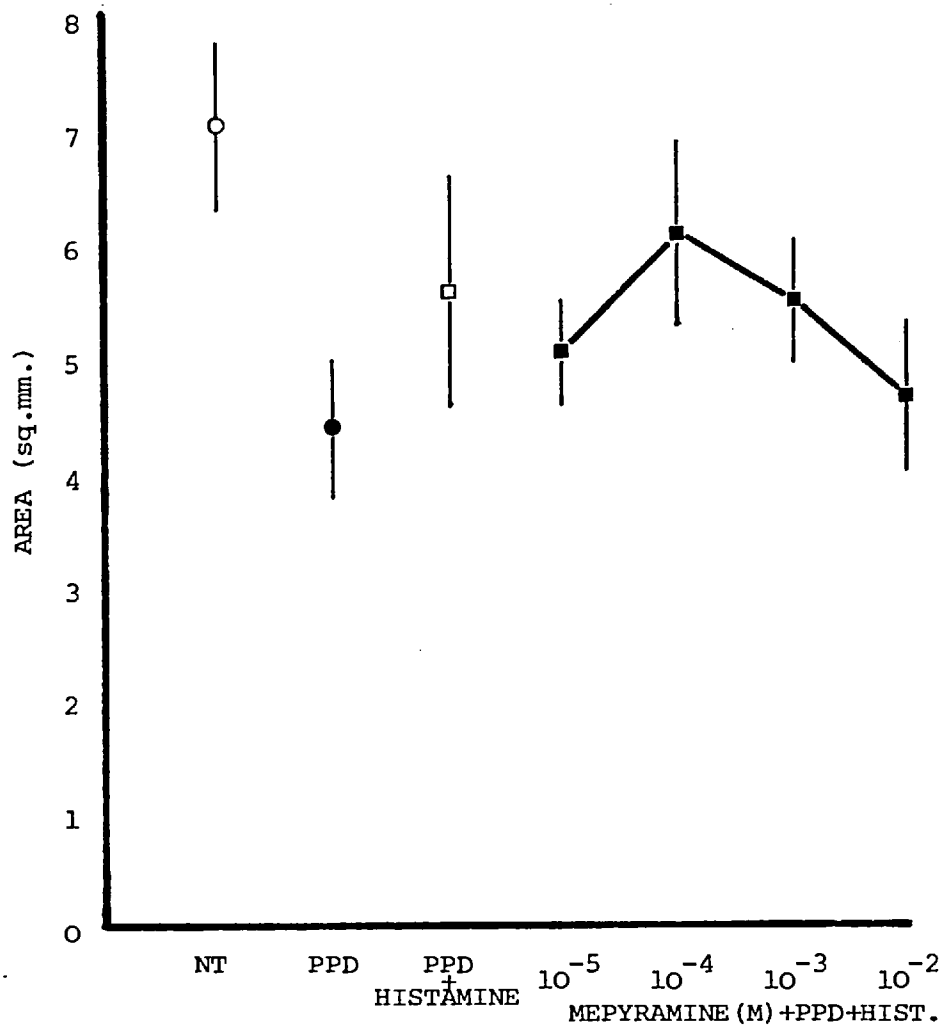

FIGURE 23 EFFECTS OF THE HISTAMINE H_1 RECEPTOR ANTAGONIST MEPYRAMINE ON THE RESPONSE TO HISTAMINE BY SENSITISED GUINEA-PIG PEC IN THE PRESENCE OF SPECIFIC ANTIGEN

PEC from BCG immunised guinea-pigs were cultured for 40 hours in the presence or absence of PPD and $\pm$ histamine (10^{-3}M) $\pm$ increasing doses of mepyramine maleate. Results expressed as area in sq.mm. Each point represents the mean $\pm$ SD of eight replicates.
○ : Cell migration in the absence of inhibitor or drugs (NT)
● : Cell migration in the presence of 2.0 ug/ml PPD.
□ : Cell migration in the presence of PPD and histamine.
■ : Cell migration in the presence of PPD + histamine and mepyramine.

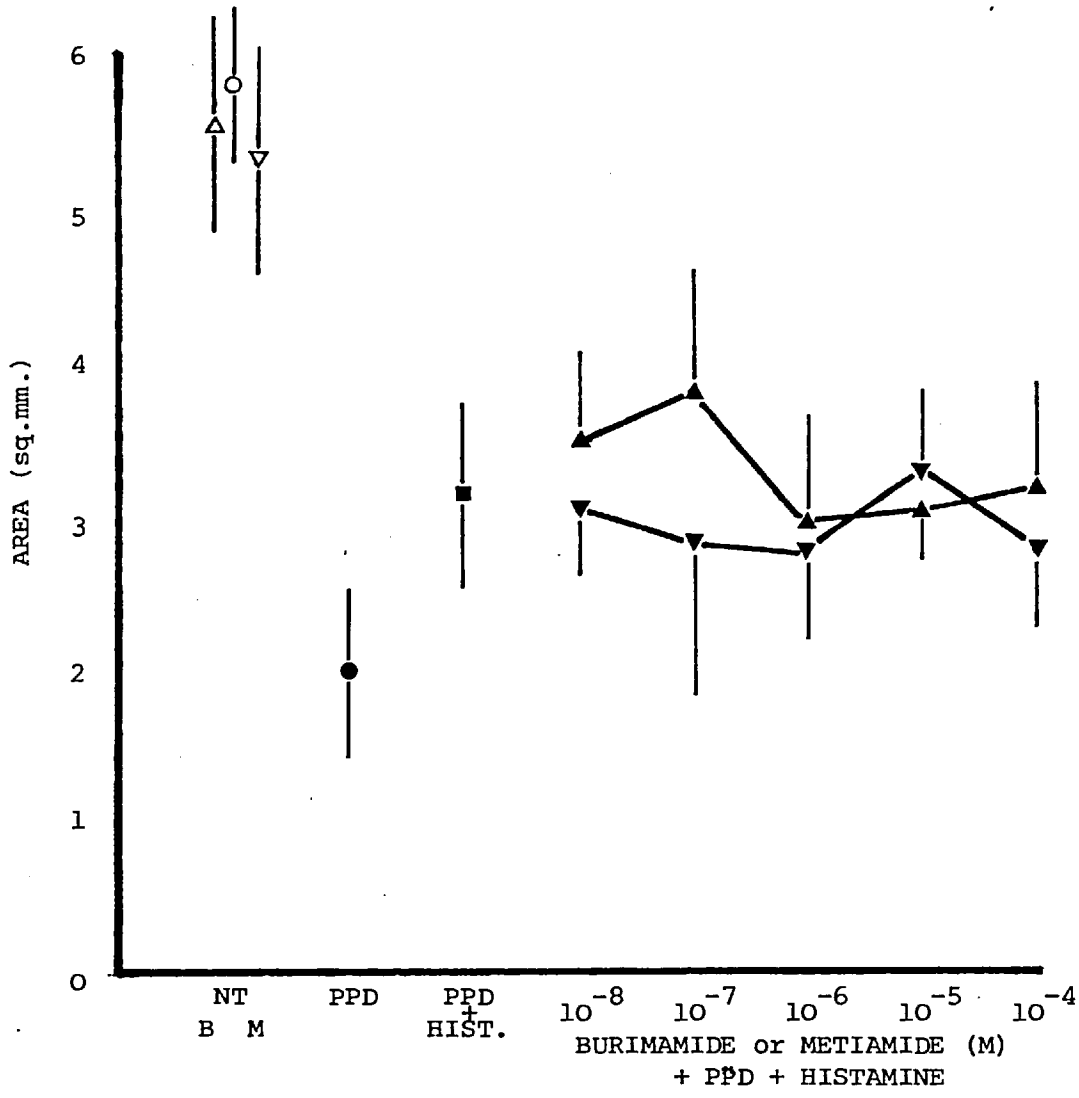

FIGURE 24 EFFECT OF THE HISTAMINE H_2 RECEPTOR ANTAGONISTS BURIMAMIDE AND METIAMIDE ON THE RESPONSE TO HISTAMINE BY SENSITISED PEC IN THE PRESENCE OF SPECIFIC ANTIGEN

PEC from BCG immunised donor guinea-pigs were cultured for 40 hours in the presence or absence of PPD and histamine $\pm$ increasing doses of burimamide or metiamide. Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of six replicate cultures.

o : Cell migration in the absence of inhibitor or drugs (NT).

$\Delta\nabla$: Cell migration in the presence of 10^{-4}M burimamide or metiamide.

● : Cell migration in the presence of 2.0 ug/ml PPD.

■ : Cell migration in the presence of PPD + histamine (10^{-3}M)

▲ : Cell migration in the presence of PPD + histamine + burimamide.

▼ : Cell migration in the presence of PPD + histamine + metiamide.

3 iii) THE EFFECTS OF GLUCOCORTICOSTEROIDS ON THE GUINEA-PIG CELL MIGRATION TEST

Steroids are known to be effective inhibitors of inflammatory responses with presumed (e.g. rheumatoid arthritis) and established (e.g. graft rejection) cellular immune aetiology. These drugs have been widely used as therapeutic agents in a variety of inflammatory or immunologically mediated diseases (Schwartz, 1968). In man, steroids suppress the migration of cells into sites of inflammation (Boggs, Athens, Cartwright and Wintrobe, 1964) and inhibit cutaneous delayed hypersensitivity responses (Gabrielson and Good, 1967). Glucocorticosteroids have also been shown to inhibit the activity of MIF on human leucocyte migration (Balow and Rosenthal, 1973).

Three anti-inflammatory steroids in general clinical use (hydrocortisone, dexamethasone and prednisolone) have been examined for their ability to modify the cell migration of guinea-pig peritoneal exudate cells in vitro. These drugs have similarly been tested for their ability to modify the migration inhibition caused by antigen or preformed lymphokine.

1) EFFECTS ON CELL MIGRATION

Addition of hydrocortisone (0.1-1000 ug/ml hydrocortisone base) to the culture medium during 40 hour culture caused a dose related enhancement of the area of cell migration with doses up to 100 ug/ml (Figure 25). A statistically significant enhancement of the response was observed with doses of 10 and 100 ug/ml (Students t-test: $p < 0.05$). A dose of 1 mg/ml caused a significant inhibition of cell migration ($p < 0.05$).

Both dexamethasone (0.01-100 ug/ml dexamethasone base) and prednisolone (0.01-100 ug/ml prednisolone base) present during 40 hour cell culture enhanced the area of cell migration (Figures 26, 27). Statistically significant dose related enhancement of migration occurred with doses from 0.1-10 ug/ml of both drugs (Students t-test: $p < 0.05$) and doses of 100 ug/ml caused some inhibition of the response.

From the data presented it is apparent that all three steroids were capable of enhancing the migration of guinea-pig peritoneal exudate cells over the dose range tested with hydrocortisone being approximately ten fold less active then either dexamethasone or prednisolone.

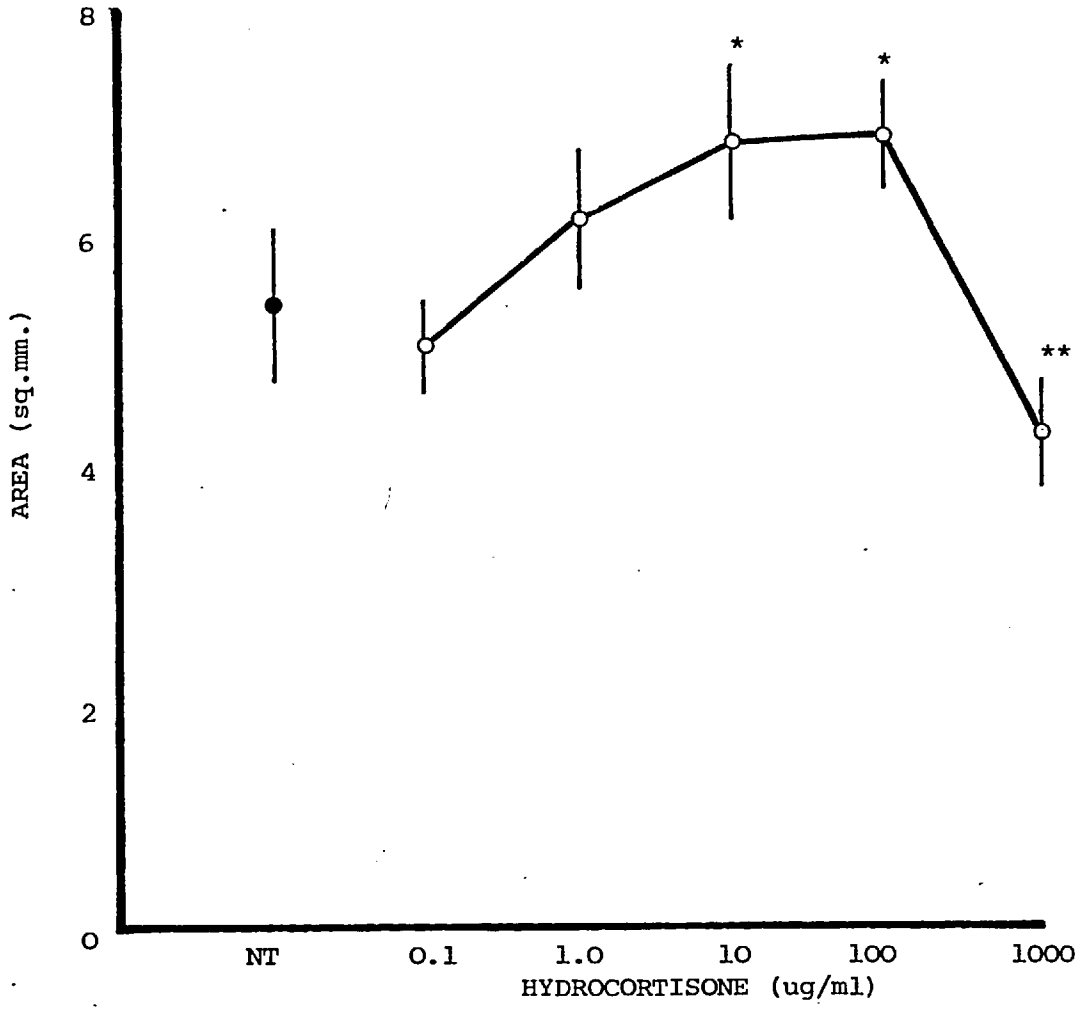

FIGURE 25 EFFECT OF INCREASING DOSES OF HYDROCORTISONE ON GUINEA-PIG PEC CAPILLARY TUBE MIGRATION

PEC from donor animals were cultured for 40 hours in the presence or absence of increasing doses of hydrocortisone acid phosphate (doses of drug expressed as ug/ml hydrocortisone). Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.
● : Cell migration in the absence of drugs (NT)
o : Cell migration in the presence of increasing doses of hydrocortisone.
* Significant enhancement of migration. Students t-test $p < 0.05$.
** Significant inhibition of migration Students t-test $p < 0.05$

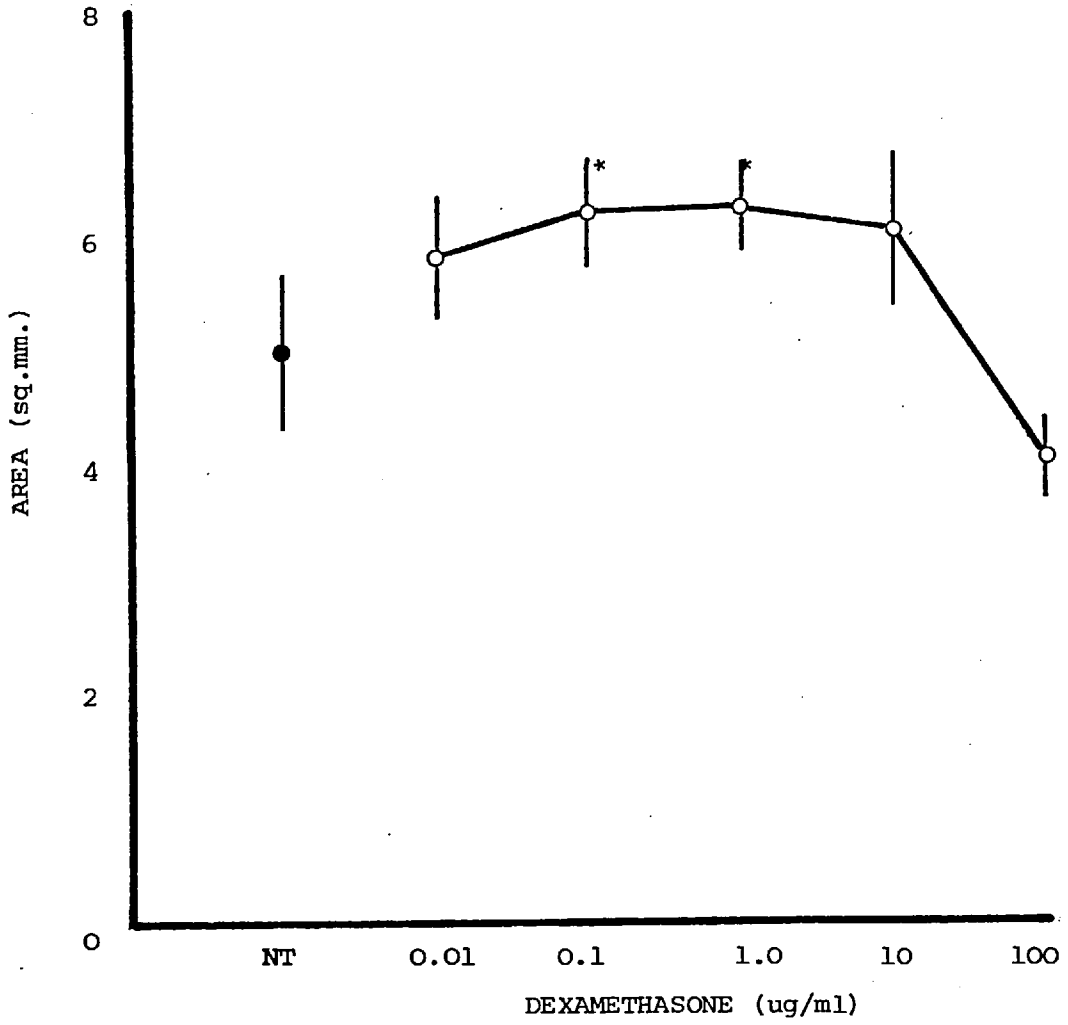

FIGURE 26 EFFECT OF INCREASING DOSES OF DEXAMETHASONE ON GUINEA-PIG CAPILLARY TUBE MIGRATION

PEC from donor animals were incubated for 40 hours in the presence or absence of increasing doses of dexamethasone sodium phosphate (doses of drug expressed as ug/ml dexamethasone). Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.
●: Cell migration in the absence of drug (NT).
o: Cell migration in the presence of increasing doses of dexamethasone.
* Significant enhancement of migration. Students t-test $p < 0.05$.

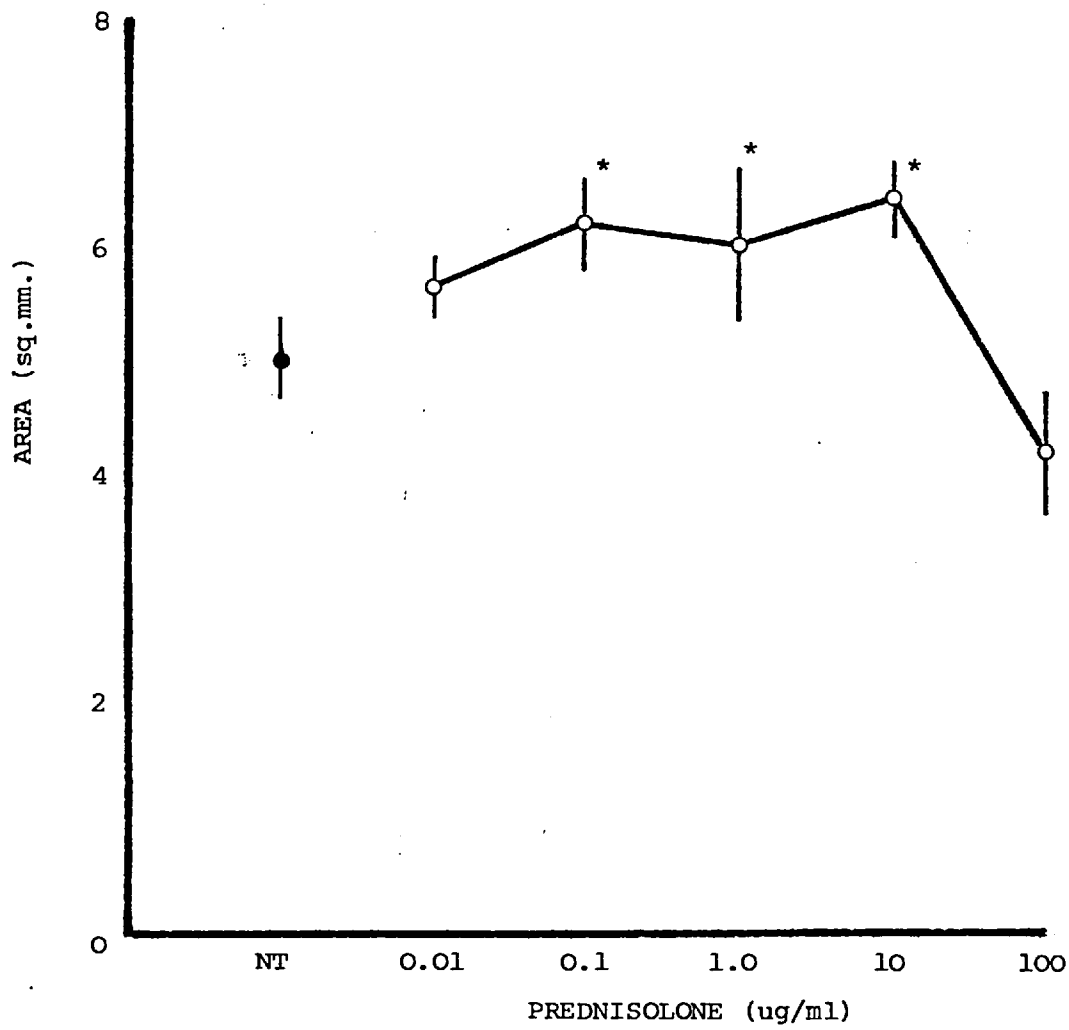

FIGURE 27 EFFECT OF INCREASING DOSES OF PREDNISOLONE ON GUINEA-PIG CAPILLARY TUBE MIGRATION

PEC from donor animals were incubated for 40 hours in the presence or absence of increasing doses of prednisolone sodium phosphate (doses of drug expressed as ug/ml prednisolone). Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.

● : Cell migration in the absence of drug (NT)

o : Cell migration in the presence of increasing doses of prednisolone.

* Significant enhancement of migration. Students t-test $p < 0.05$.

2) EFFECTS OF STEROIDS ON ANTIGEN INDUCED MACROPHAGE MIGRATION INHIBITION

The three steroids were tested using the same assay system for their ability to affect the inhibition of cell migration caused by antigen. Guinea-pig peritoneal exudate cells from animals immunised to BCG four weeks previously were cultured for 40 hours in the presence of 2.0 ug/ml PPD (ID_{50}: Table 3) and increasing doses of steroid. Doses of hydrocortisone base ranging from 1.0 - 1000 ug/ml caused a statistically significant reduction in the cell migration inhibition caused by antigen (Students t-test: $p < 0.05$). The maximum reduction occurred with a dose of 100 ug/ml (Figure 28). Both dexamethasone and prednisolone caused a reduction in the inhibitory effect of antigen over the dose range 0.01 - 100 ug/ml for dexamethasone and 0.01 - 10 ug/ml for prednisolone with the maximum inhibition of the response occurring with 10.0 ug/ml and 1.0 ug/ml respectively (Students t-test: $p < 0.05$) (Figures, 29, 30).

The effective dose ranges for all three drugs were similar to those observed for the enhancement of cell migration in the absence of antigen and as with this latter response hydrocortisone was the least active of the three steroids examined.

In order to further quantify the inhibitory effect of glucocorticosteroids on the cell migration response of sensitised cells to antigen a range of steroid doses (0.1-10 ug/ml) were tested for their ability to affect the cell migration inhibition response of PEC from BCG sensitised animals to increasing doses of antigen (PPD). Hydrocortisone (0.1-10 mg/ml hydrocortisone base) added to cultures containing increasing doses of PPD (0.3-10 ug/ml) caused a dose related shift in the inhibitory dose response curve to PPD (Figure 31). At each of the steroid dose levels potency ratio estimates were calculated with respect to the inhibitory effect in the absence of drug. Potency ratio estimates and 95% confidence limits for these parallel line assay data have been tabulated (Table 8).

The effect of dexamethasone and prednisolone were similarly estimated. Doses of each drug ranging from 0.1-10 ug/ml were tested (Figures 32, 33) and the potency ratio estimates calculated (Table 8). These results demonstrate that all three steroids tested caused a dose related reduction in the ability of antigen to inhibit the cell migration of specifically

sensitised cells. From a comparison of the drug potencies (Table 8) it can be seen that the relative activities were hydrocortisone<dexamethasone<prednisolone. The ability of both dexamethasone and prednisolone not only to abolish the inhibitory effects of antigen at low doses but also to increase the area of cell migration relative to control responses implied that the inhibitory effect of these drugs was not due to an action on the antigen response. An explanation for the inhibition of the antigen response may be derived from the results shown in Figures 25-27 where the steroids enhanced the cell migration of PEC in culture. From these results it may be concluded that the steroids act by enhancing cell migration rather than by inhibiting the effect of antigen providing a "physiological" rather than "pharmacological" antagonism.

3) EFFECTS OF STEROIDS ON LYMPHOKINE INDUCED MACROPHAGE MIGRATION INHIBITION

The ability of steroids to modify the response of guinea-pig PEC to lymphokine has also been tested. Doses of hydrocortisone, dexamethasone and prednisolone (all 1.0 ug/ml) added with increasing doses of preformed lymphokine material (100-400 ug/ml) during 40 hour cell culture caused substantial inhibition of the inhibitory lymphokine dose response line (Figure 34). All three steroids were approximately equiactive at the doses tested with relative potency shifts for hydrocortisone of 1.72; dexamethasone, 1.82; and prednisolone 1.87.

The relative activities were in the same order as for inhibition of the antigen response, however, the activity of hydrocortisone appeared to be considerably closer to that of the other two drugs in its ability to inhibit the response to preformed lymphokine.

The ability of the steroids to inhibit the cell migration inhibitory effect of preformed lymphokine implied that the drugs were acting upon the target macrophage population rather than the lymphocyte population responding to specific antigen. This result is consistent with the non-immunological role of steroid suppression of cell migration inhibition in vitro proposed with respect to their action on the antigen induced cell migration inhibition.

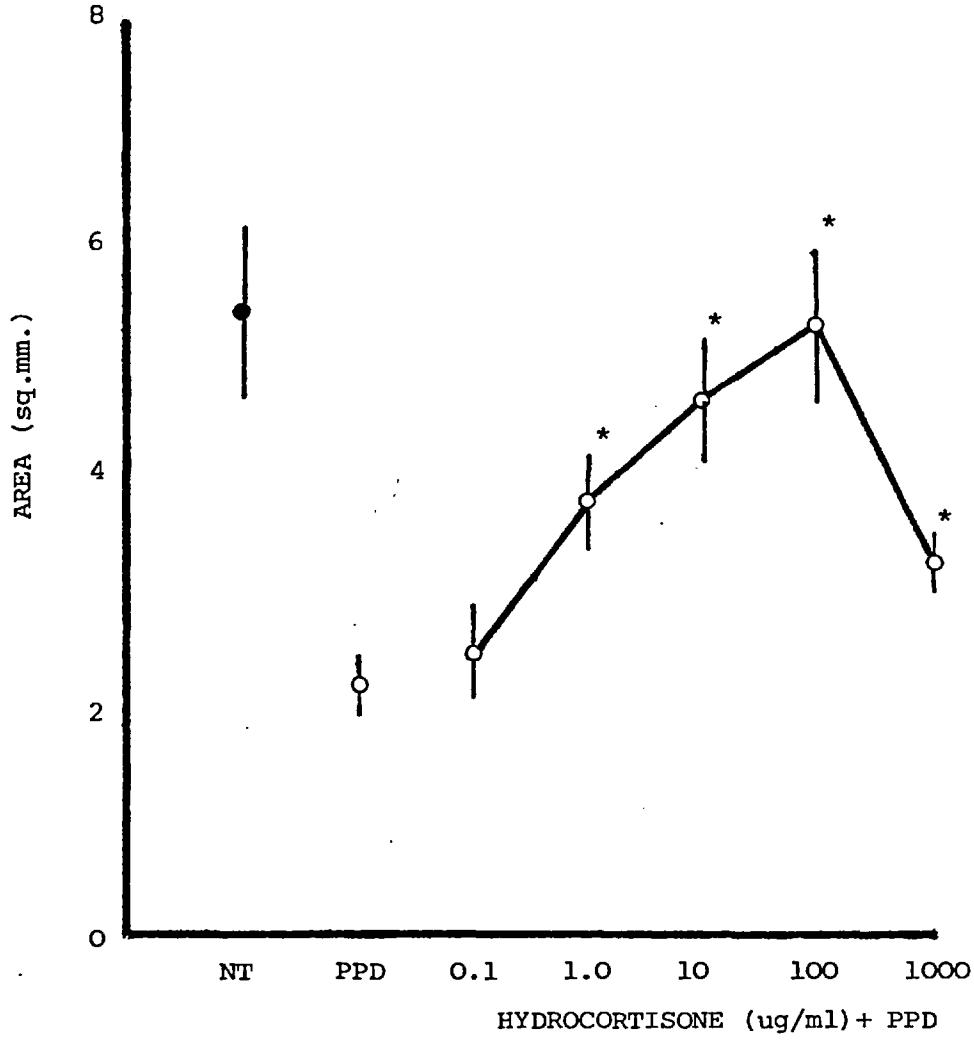

FIGURE 28 EFFECT OF INCREASING DOSES OF HYDROCORTISONE ON THE INHIBITION OF TUBERCULIN SENSITISED GUINEA-PIG CELLS CAUSED BY PPD

PEC from animals sensitised to BCG were cultured for 40 hours in the presence of specific antigen (PPD 2.0 ug/ml) and increasing doses of hydrocortisone acid phosphate (expressed as ug/ml hydrocortisone). Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.

● : Cell migration in the absence of antigen or drug (NT).

o : Cell migration in the presence of PPD$^{\pm}$ hydrocortisone.

* Significant reduction in inhibition. Students t-test $p < 0.05$.

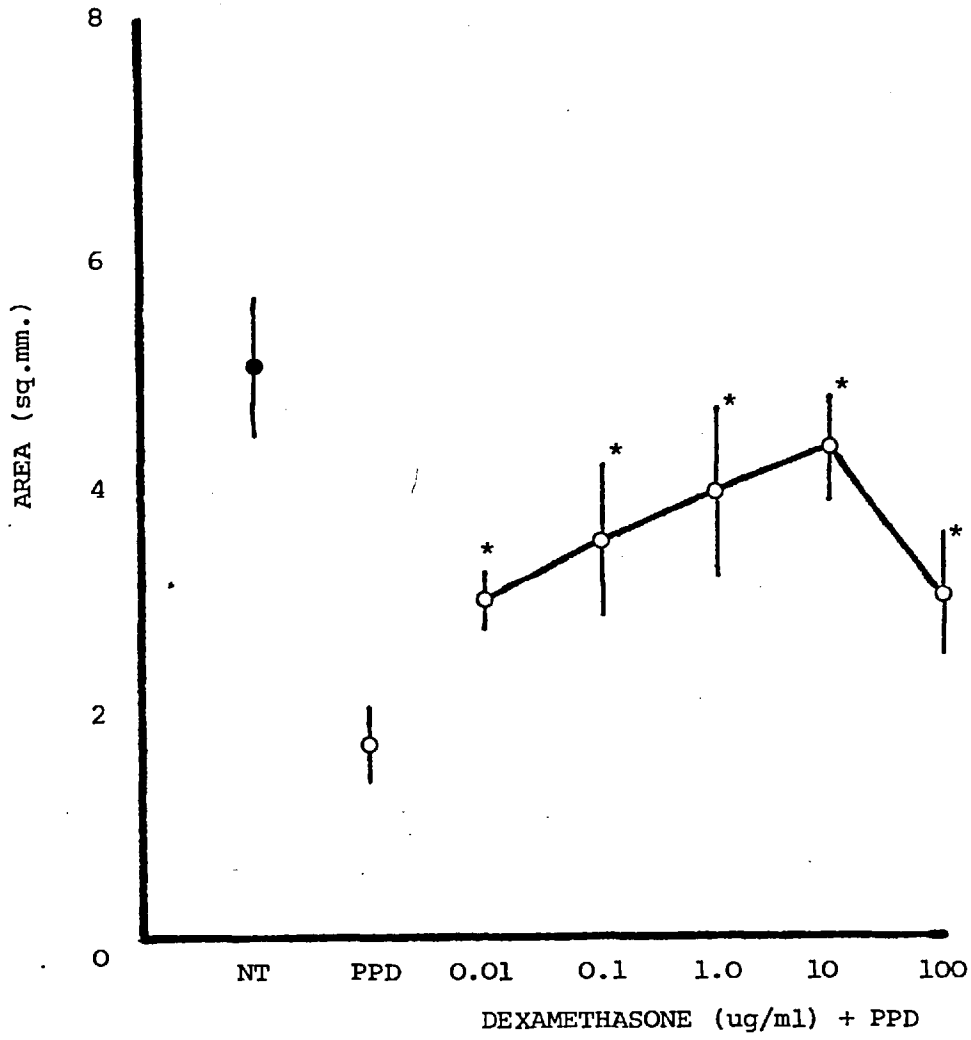

FIGURE 29 EFFECT OF INCREASING DOSES OF DEXAMETHASONE ON THE INHIBITION OF TUBERCULIN SENSITISED GUINEA-PIG CELLS CAUSED BY PPD

PEC from animals sensitised to BCG were cultured for 40 hours in the presence of specific antigen (PPD 2.0 ug/ml) and increasing doses of dexamethasone acid phosphate (expressed ug/ml dexamethasone). Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.

● : Cell migration in the absence of antigen or drug (NT).

o : Cell migration in the presence of PPD$\pm$ dexamethasone.

* Significant reduction in inhibition. Students t-test $p < 0.05$

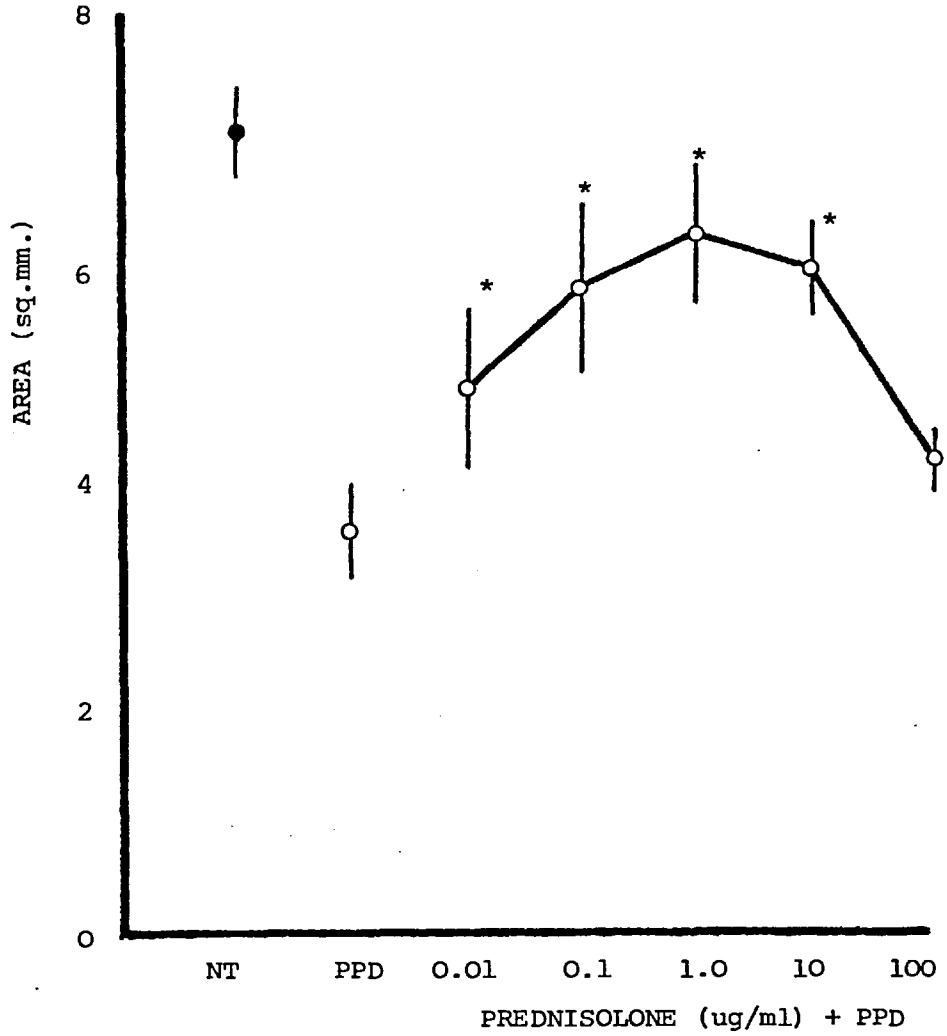

FIGURE 30 EFFECT OF INCREASING DOSES OF PREDNISOLONE ON THE INHIBITION OF TUBERCULIN SENSITISED GUINEA-PIG CELLS CAUSED BY PPD

PEC from animals sensitised to BCG were cultured for 40 hours in the presence of specific antigen (PPD 2.0 ug/ml) and increasing doses of prednisolone acid phosphate (expressed ug/ml prednisolone). Results expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.
● : Cell migration in the absence of antigen or drug (NT).
o : Cell migration in the presence of PPD$^{\pm}$ prednisolone.
* Significant reduction in inhibition. Students t-test $p < 0.05$.

TABLE 8 INHIBITION OF GUINEA-PIG CELL MIGRATION RESPONSE TO SPECIFIC ANTIGEN. DOSE RATIO ESTIMATES DERIVED FROM PARALLEL LINE ASSAY DATA FOR INCREASING DOSES OF SPECIFIC ANTIGEN (PPD) IN THE PRESENCE OF INCREASING DOSES OF THREE STEROIDS

	Dose of drug (ug/ml)	Dose ratio* (Ratio in the absence of drug taken as 1.0)
Hydrocortisone	0	1.0
	0.1	1.73
	0.3	3.82
	1.0	7.35
	3.0	14.71
	10.0	25.64
Dexamethasone	0	1.0
	0.1	5.33
	0.3	16.0
	1.0	27.66
	3.0	96.5
	10.0	161.0
Prednisolone	0	1.0
	0.1	4.37
	0.3	18.45
	1.0	40.0
	3.0	110.5
	10.0	237.5

* Each figure represents the mean dose ratio from three separate experiments (total n = 18) performed on different occasions

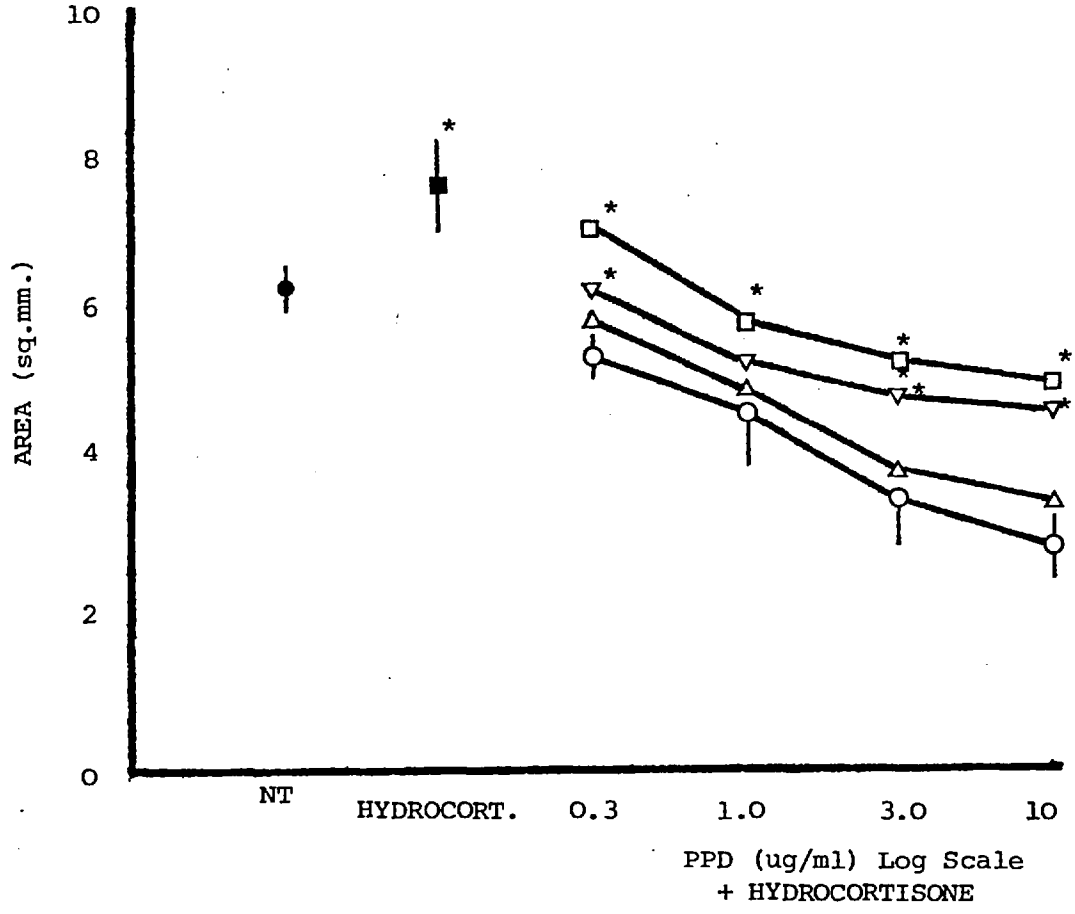

FIGURE 31 EFFECT OF SEVERAL DOSES OF HYDROCORTISONE ON THE RESPONSE OF TUBERCULIN SENSITISED GUINEA-PIG PEC TO INCREASING DOSES OF PPD

PEC from donor animals were cultured for 40 hours in the presence of increasing doses of PPD ± hydrocortisone. Results were expressed as area in sq.mm. Each point represents the mean (± SD where shown) of six replicate cultures.

● : Cell migration in the absence of antigen or drug (NT).

■ : Cell migration in the presence of hydrocortisone (1.0 ug/ml).

○ : Cell migration in the presence of increasing doses of PPD.

: Cell migration in the presence of increasing doses of PPD + hydrocortisone

△:0.1 ug/ml; ▽:0.3 ug/ml; □:1.0 ug/ml.

* Significant enhancement of migration. Students t-test $p < 0.05$.

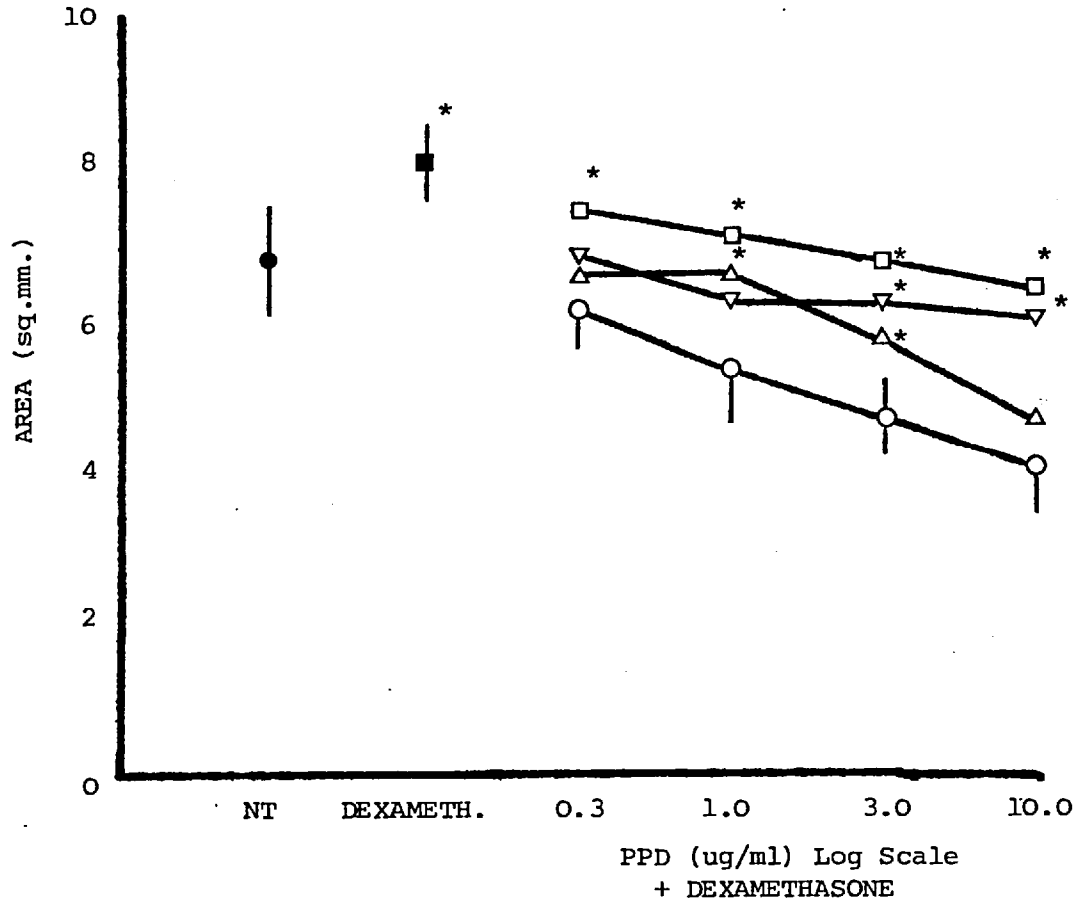

FIGURE 32 EFFECT OF SEVERAL DOSES OF DEXAMETHASONE ON THE RESPONSE OF TUBERCULIN SENSITISED GUINEA-PIG PEC TO INCREASING DOSES OF PPD

PEC from donor animals were cultured for 40 hours in the presence of increasing doses of PPD ± dexamethasone. Results were expressed as area in sq.mm. Each point represents the mean (± SD where shown) of six replicate cultures.

● : Cell migration in the absence of antigen or drug (NT).

■ : Cell migration in the presence of dexamethasone (1.0 ug/ml).

o : Cell migration in the presence of increasing doses of PPD.

: Cell migration in the presence of increasing doses of PPD + dexamethasone △ : 0.1 ug/ml; ▽ : 0.3 ug/ml; □ : 1.0 ug/ml.

* Significant enhancement of migration. Students t-test $p < 0.05$.

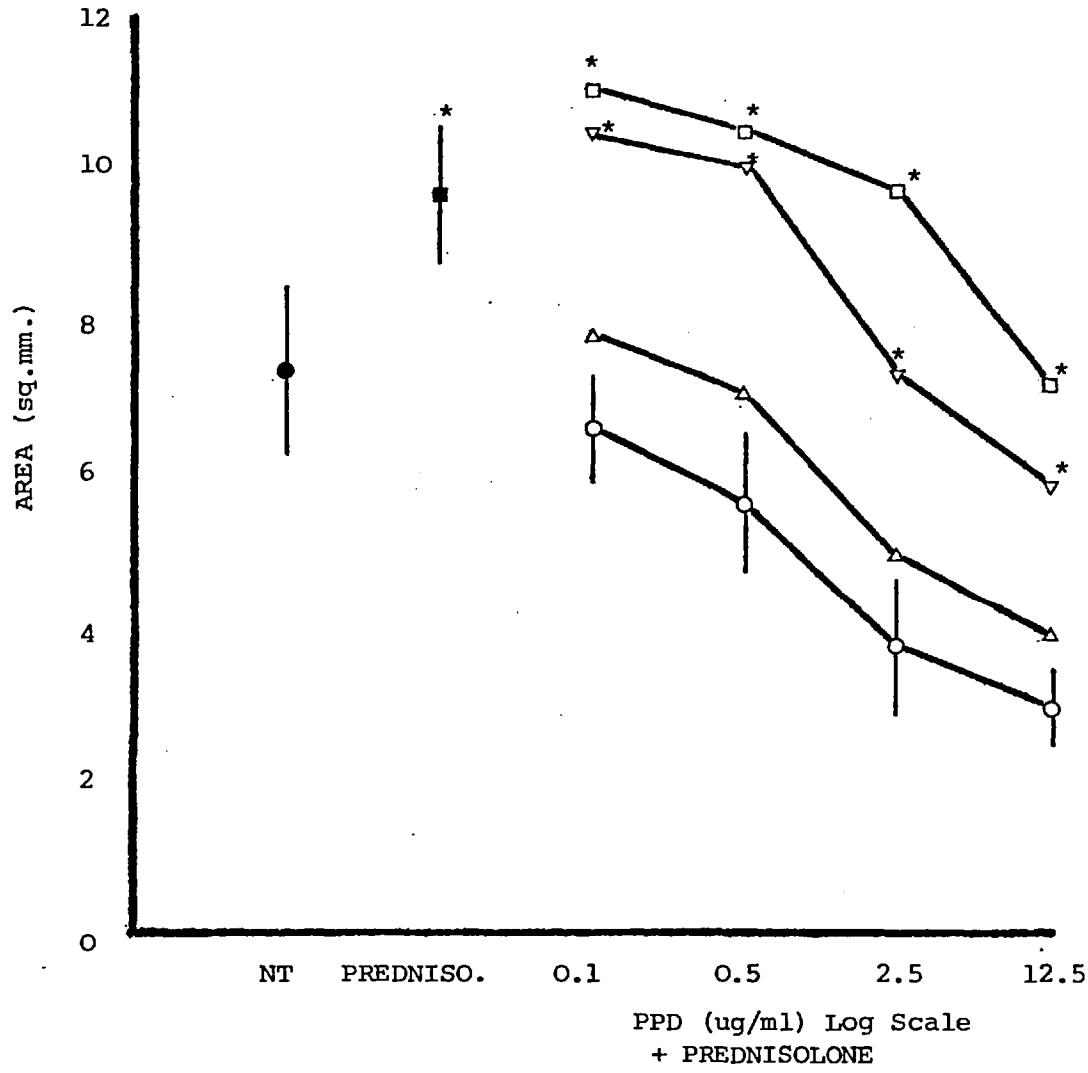

FIGURE 33 EFFECT OF SEVERAL DOSES OF PREDNISOLONE ON THE RESPONSE OF TUBERCULIN SENSITISED GUINEA-PIG PEC TO INCREASING DOSES OF PPD

PEC from donor animals were cultured for 40 hours in the presence of increasing doses of PPD ± prednisolone. Results were expressed as area in sq.mm. Each point represents the mean (± SD where shown) of six replicate cultures.

● : Cell migration in the absence of antigen or drug (NT).

■ : Cell migration in the presence of prednisolone (1.0 ug/ml).

○ : Cell migration in the presence of increasing doses of PPD

: Cell migration in the presence of increasing doses of PPD + prednisolone △:0.1 ug/ml; ▽ : 1.0 ug/ml; □:10.0 ug/ml.

* Significant enhancement of migration. Students t-test $p < 0.05$.

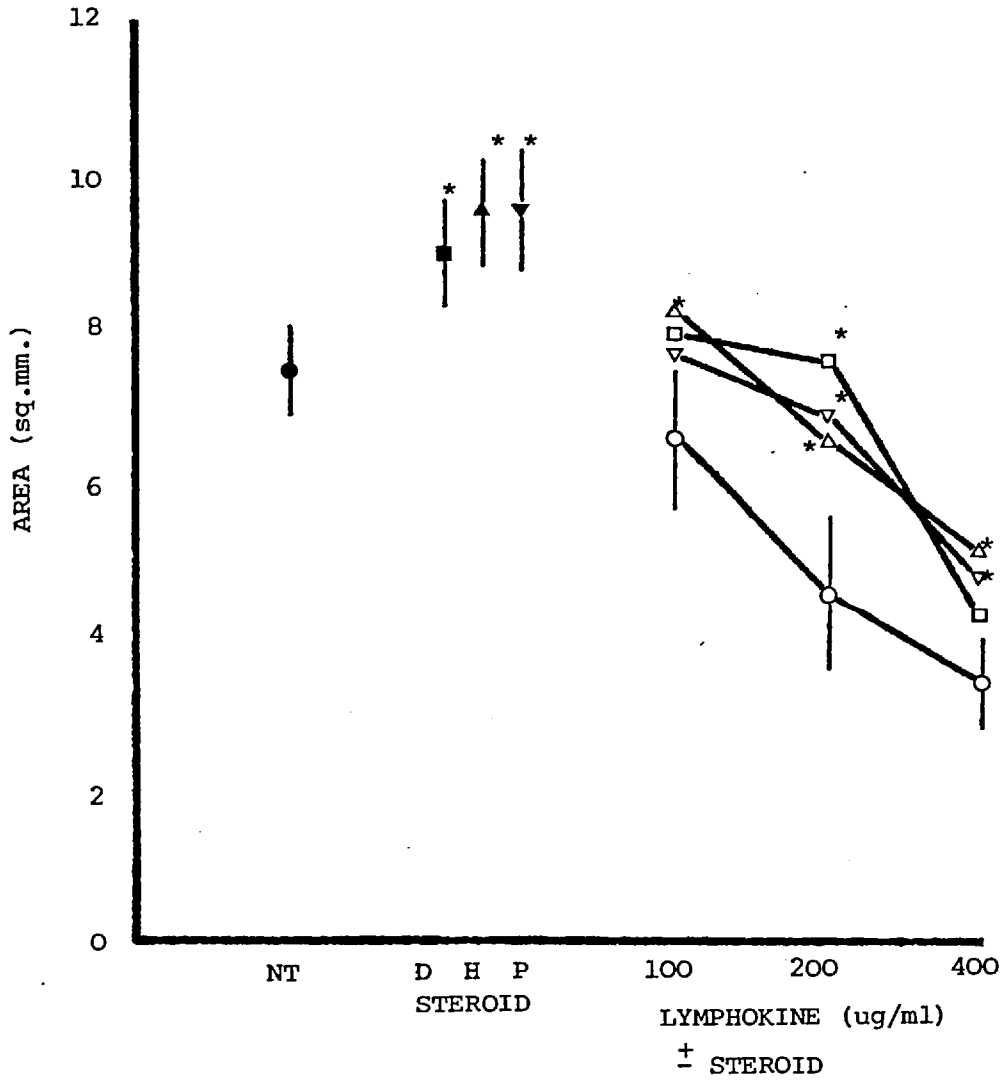

FIGURE 34 EFFECT OF GLUCOCORTICOIDS ON THE CELL MIGRATION INHIBITION RESPONSE OF GUINEA-PIG PEC TO PREFORMED LYMPHOKINE

PEC from donor animals were cultured for 40 hours in the presence of increasing doses of lyophilised preformed lymphokine ± glucocorticoid. Results were expressed as area of cell migration in sq.mm. Each point represents the mean ± SD of six replicate cultures.
Solid symbols: Cell migration in the absence of antigen or drug (NT) (●) and in the presence of 1.0 ug/ml hydrocortisone (▲) dexamethasone (■) and prednisolone (▼).
Open symbols: Cell migration in the presence of increasing doses of LK (○) and with 1.0 ug/ml hydrocortisone (△) dexamethasone (□) and prednisolone (▽).
* Significant enhancement of migration. Students t-test $p < 0.05$.

3 iii) PROSTAGLANDINS

In recent years prostaglandins have been widely considered as mediators of both acute and chronic inflammation, being both algesic and pyretic. The likelihood of such a role extending to include pro-inflammatory actions was enhanced by the findings of Vane and co-workers who showed that non-steroidal anti-inflammatory drugs may exert their influence via inhibition of prostaglandin biosynthesis (Vane, 1971: Smith & Willis, 1971; Ferriera, et al., 1971). Prostaglandins have been detected in exudates from a variety of inflammatory lesions, both experimental (e.g. carageenan oedema, ocular inflammation and monoarticular arthritis) and pathological (e.g. rheumatoid arthritis) (Anderson et al., 1971; Eakins et al., 1972; Blackham et al., 1974 and Robinson et al., 1973). In vitro E-type prostaglandins have been shown to affect functions of cell types involved in the inflammatory response. For instance, PGE_1 is chemotactic for rabbit polymorphonuclear leucocytes (Kaley & Weiner, 1971) and PGE's are potent stimulators of intracellular cAMP within both lymphocytes (Smith et al., 1971) and macrophages (Gemsa, Seitz, Kramer, Till & Resch, 1975). Such data has been taken as an indication that PG's may play a role as modulators of inflammatory immune responses of lymphocytes to PHA (Smith et al., 1971)

Such a background has led to the current study of the possible interactions between prostaglandins (particularly of the E-series) and the generation and action of mediators of cellular immunity. The effects of PG's have been tested on the cell migration inhibition, lymphocyte transformation and increased vascular permeability assays for cell mediated immune responses.

1) EFFECTS OF PROSTAGLANDINS ON CELL MIGRATION

Doses of PGE_1 (0.001-10 ug/ml); PGE_2 (0.001-10 ug/ml); PGA_1 (0.01-1.0 ug/ml) and $PGF_{2\alpha}$(0.01-1.0 ug/ml) have been tested for their effects on guinea-pig peritoneal exudate cell migration. Drugs were present throughout the culture period (40 hours) and in no case were significant effects seen with doses of prostaglandins up to 1.0 ug/ml (Figure 35). Doses of 10 ug/ml PGE_1 and PGE_2 caused a slight reduction in the area of cell migration and all subsequent experiments were carried out with doses of prostaglandins below 10 ug/ml.

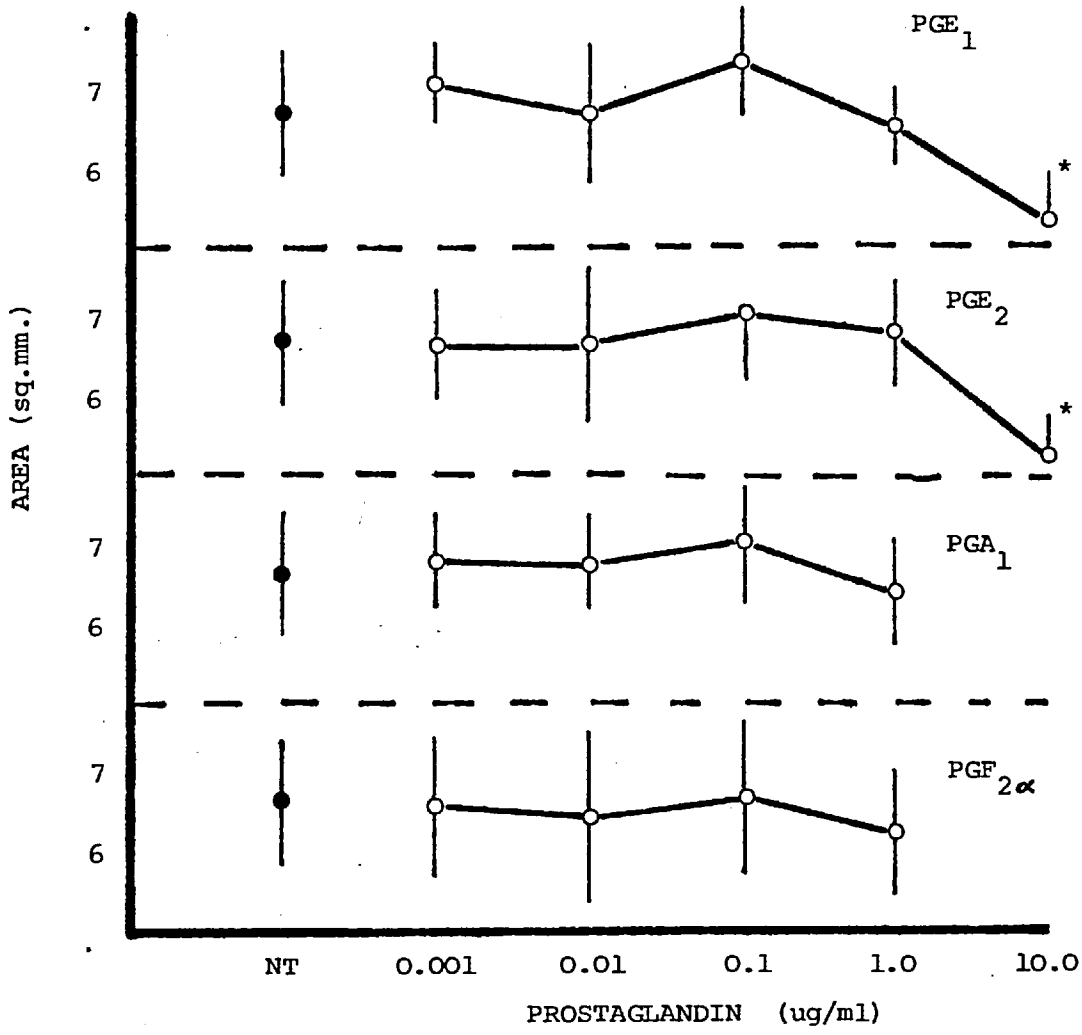

FIGURE 35 EFFECT OF INCREASING DOSES OF PROSTAGLANDINS ON THE CAPILLARY TUBE MIGRATION OF GUINEA-PIG CELLS

PEC from donor guinea-pigs were cultured for 40 hours in the presence of increasing doses of several PGs. Results are expressed as area of migration in sq.mm. Each point represents the mean ± SD of eight replicate cultures.
● : Cell migration in the absence of PG.
o : Cell migration in the presence of PG.
* Significant inhibition of migration. Students t-test $p < 0.05$.

2) EFFECT OF PROSTAGLANDINS ON ANTIGEN INDUCED MACROHPAGE MIGRATION INHIBITION

In initial experiments the effect of four prostaglandins (PGE_1, E_2, A_1 and $F_{2\alpha}$)were examined. Drugs at doses of 0.001-1.0 ug/ml were added to migration chambers containing guinea-pig PEC sensitised to tuberculin in the presence of a fixed dose of specific antigen (PPD: 2.0 ug/ml). Both PGE_1 and PGE_2 caused a dose related reduction in the response to antigen, maximal inhibition occurring with doses of 1.0 ug/ml (Figures 36, 37). PGA_1 had a slight inhibitory effect at 1.0 ug/ml and $PGF_{2\alpha}$ was inactive.

The ability of E-type prostaglandins to inhibit the cell migration response to specific antigen was further tested by adding PGE_1 (0.1 and 1.0 ug/ml) and PGE_2 (0.01-1.0 ug/ml) to cultures containing increasing doses of specific antigen. PGE_1 caused a dose related parallel shift (by analysis of variance the deviations from parallelism were not significant. $p > 0.05$) in the migration inhibitory dose response lines obtained with specific antigen (Figure 38). The relative potency shifts for PGE_1, 0.1 and 1.0 ug/ml were 3.60 and 7.34 respectively as calculated from 3 + 3 potency ratio analysis. PGE_2 caused a similar reduction in the response to antigen with doses of drugs ranging from 0.01-1.0 ug/ml (Figure 39). As with PGE_1 there was no significant deviation from parallelism (by analysis of variance $p > 0.05$) and potency ratio estimates with 95% confidence limits for the doses administered are shown in Table 9 along with similar data for PGE_1. From Table 9 it appears that PGE_2 is a slightly more potent inhibitor of antigen induced cell migration inhibition than PGE_1.

3) EFFECT OF E-TYPE PROSTAGLANDINS ON LYMPHOCYTE TRANSFORMATION AND INCREASED VASCULAR PERMEABILITY

The ability of E-type prostaglandins to inhibit antigen induced cell migration inhibition <u>in vitro</u> implies a possible role for these mediators of inflammation in cell mediated immune responses. The effects of E-type prostaglandins on antigen specific lymphocyte transformation and increased vascular permeability have therefore been assessed.

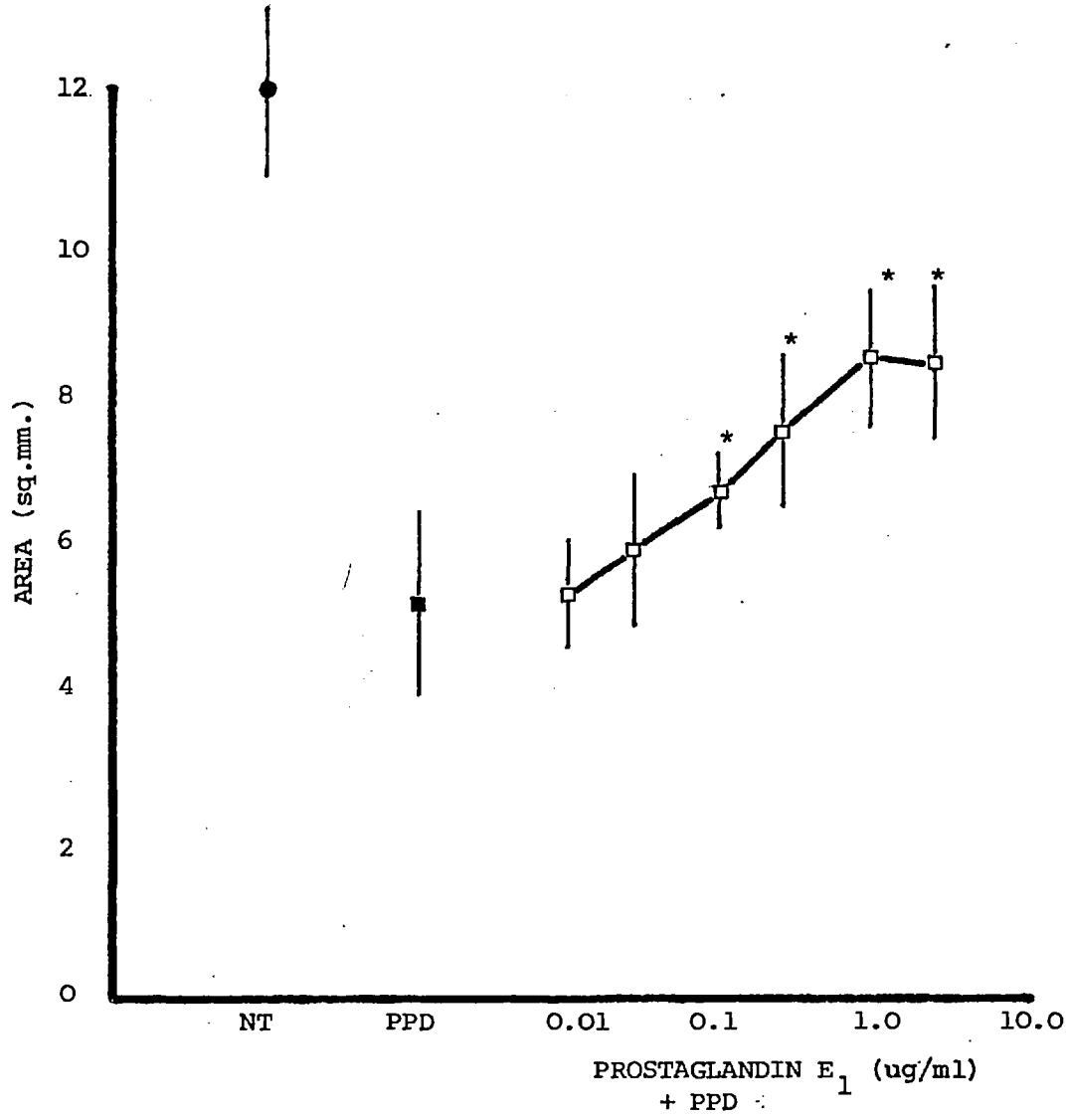

FIGURE 36 EFFECT OF INCREASING DOSES OF PGE_1 ON THE ANTIGEN INDUCED INHIBITION OF GUINEA-PIG CAPILLARY TUBE CELL MIGRATION

PEC from tuberculin sensitive donor animals were incubated for 40 hours in the presence of specific antigen (PPD 2.0 ug/ml) and increasing doses of PGE_1. Results are expressed as area of migration in sq.mm. Each point represents the mean $\pm$ SD of eight replicate cultures.

● : Cell migration in the absence of antigen or PGE_1 (NT).

■ : Cell migration in the presence of PPD (2.0 ug/ml).

□ : Cell migration in the presence of PPD and PGE_1.

* Significant reduction in inhibition. Students t-test $p < 0.05$.

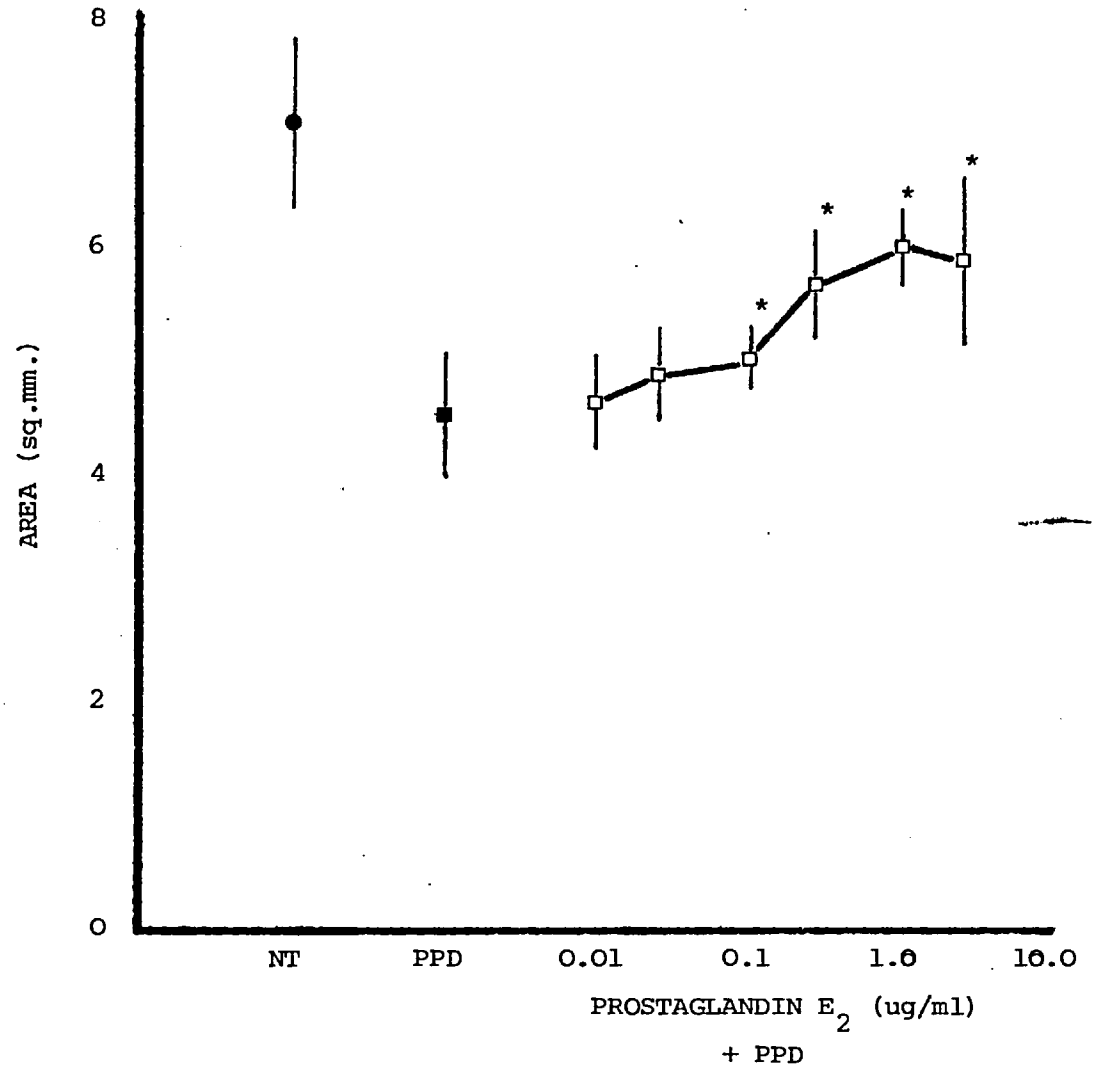

FIGURE 37 EFFECT OF INCREASING DOSES OF PGE_2 ON THE ANTIGEN INDUCED INHIBITION OF GUINEA-PIG CAPILLARY TUBE CELL MIGRATION

PEC from tuberculin sensitive donor animals were incubated for 40 hours in the presence of specific antigen (PPD 2.0 ug/ml) and increasing doses of PGE_2. Results are expressed as area of migration in sq.mm. Each point represents the mean ± SD of eight replicate cultures.

● : Cell migration in the absence of antigen or PGE_2 (NT).

■ : Cell migration in the presence of PPD (2.0 ug/ml).

□ : Cell migration in the presence of PPD and PGE_2.

* Significant reduction in inhibition. Students t-test $p < 0.05$.

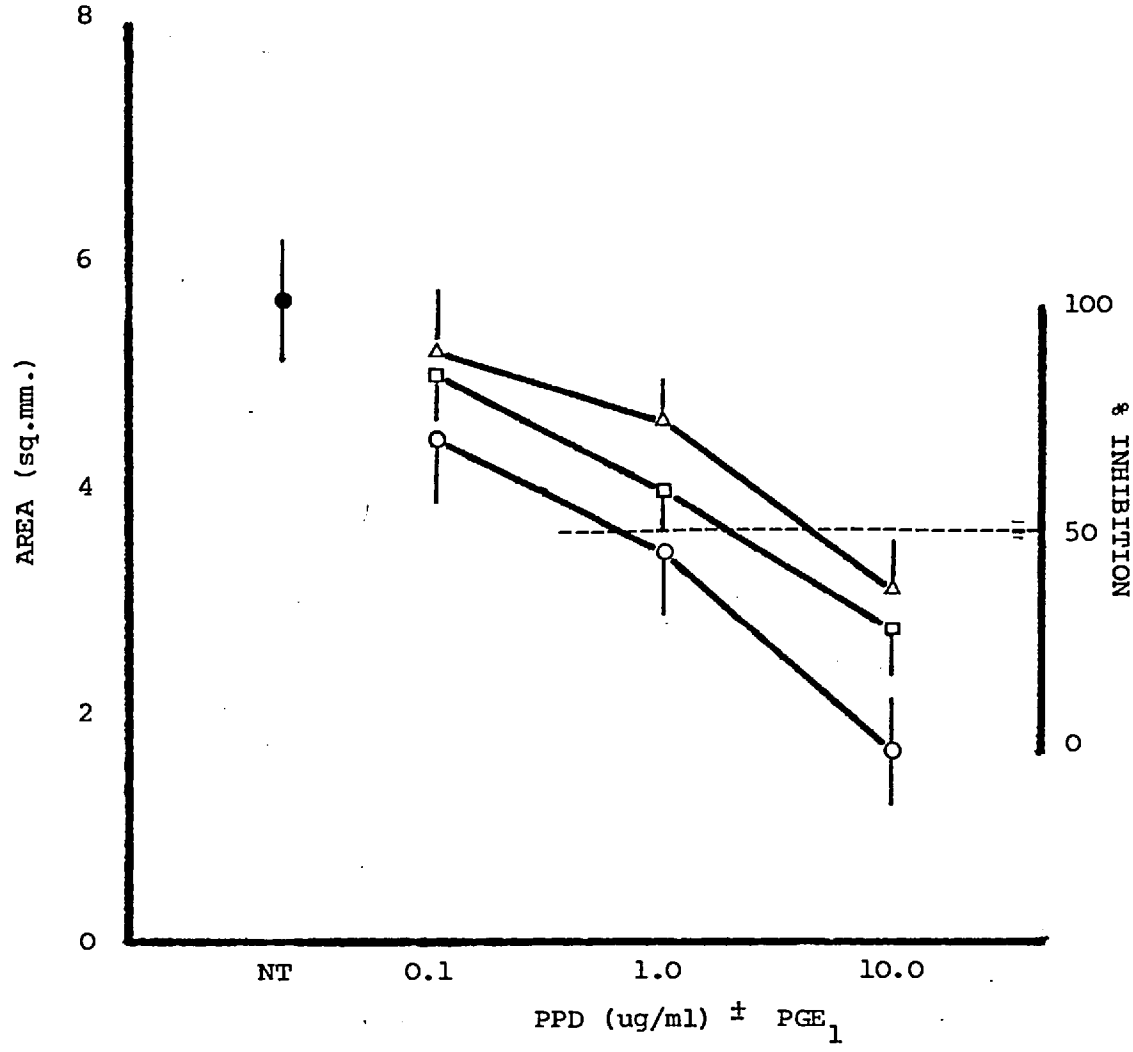

FIGURE 38 EFFECT OF INCREASING DOSES OF PGE_1 ON THE INHIBITION OF GUINEA-PIG CELL MIGRATION CAUSED BY INCREASING AMOUNTS OF SPECIFIC ANTIGEN

PEC from tuberculin sensitive donor animals were cultured for 40 hours in the presence of increasing doses of specific antigen (PPD) and varying doses of PGE_1. Results are expressed as area of migration in sq.mm. and as % of the inhibition caused by the highest dose of antigen alone. Each point represents the mean ± SD of six replicate cultures. Potency ratio estimates for the inhibition of the antigen response by PGE_1 are shown in Table 8.

● : Cell migration in the absence of antigen or PGE_1 (NT).

○ : Cell migration in the presence of antigen.

□ : Cell migration in the presence of antigen and 0.1 ug/ml PGE_1.

△ : Cell migration in the presence of antigen and 1.0 ug/ml PGE_1.

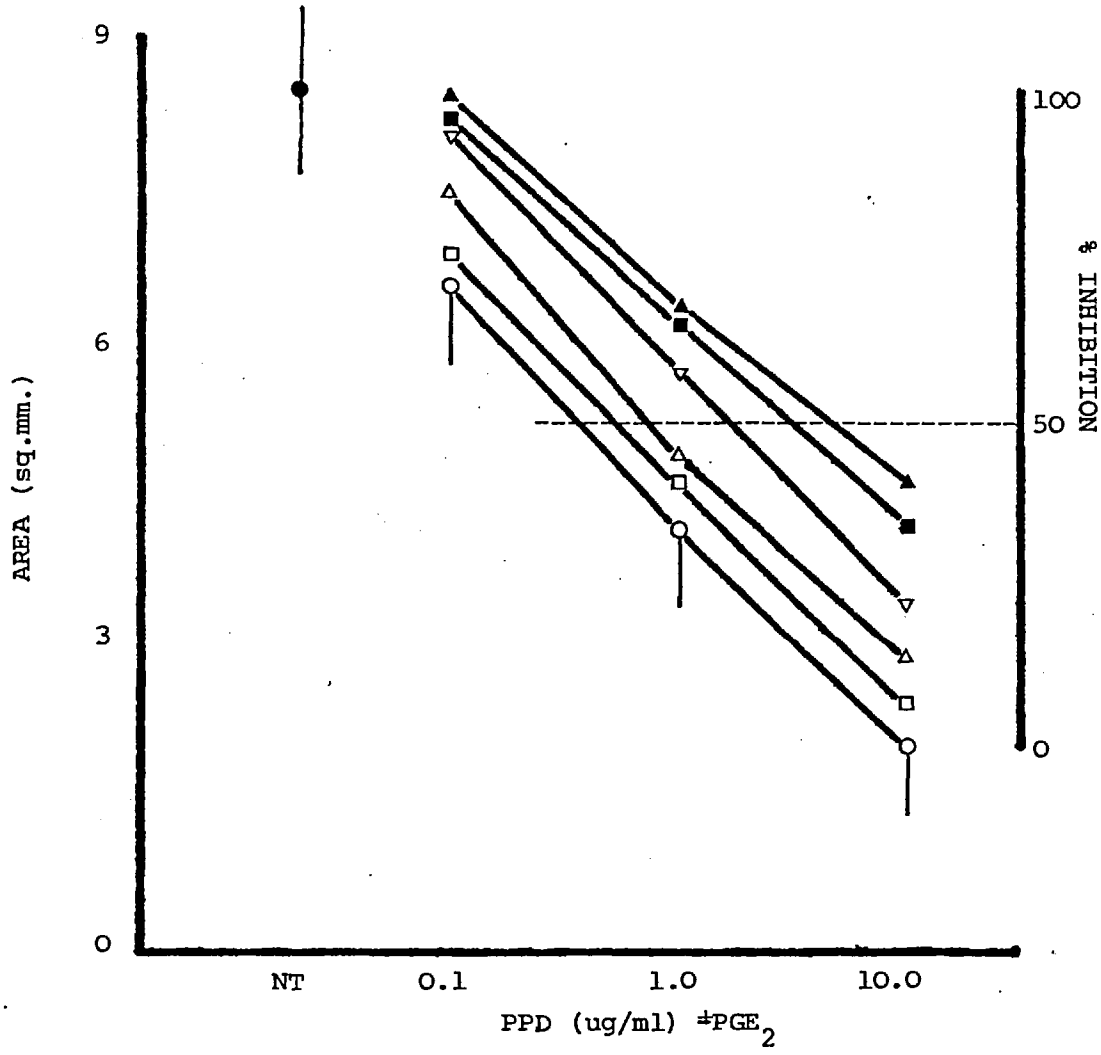

FIGURE 39 EFFECT OF INCREASING DOSES OF PGE_2 ON THE INHIBITION OF GUINEA-PIG CELL MIGRATION CAUSED BY INCREASING AMOUNTS OF SPECIFIC ANTIGEN

PEC from tuberculin sensitive donor animals was cultured for 40 hours in the presence of increasing doses of specific antigen (PPD) and varying doses of PGE_2. Results are expressed as area of migration in sq.mm. and as % of the inhibition caused by the highest dose of antigen alone. Each point represents the mean ± SD of six replicate cultures. Potency ratio estimates for the inhibition of the antigen response by PGE_2 are shown in Table 8.

● : No antigen or PGE_2 (NT).
○ : Antigen alone.
□ : Antigen + PGE_2 0.01 ug/ml.
△ : Antigen + PGE_2 0.03 ug/ml.
▽ : Antigen + PGE_2 0.1 ug/ml.
■ : Antigen + PGE_2 0.3 ug/ml.
▲ : Antigen + PGE_2 1.0 ug/ml.

TABLE 9 INHIBITION OF ANTIGEN STIMULATED MMI BY E-TYPE PGs

Dose of drug (n = 6)	Potency ratio (95% confidence limits)
PGE_1	
0.1	3.60 (4.73, 2.76)
1.0	7.34 (9.10, 6.23)
PGE_2	
0.01	1.56 (1.89, 1.23)
0.03	1.86 (2.04, 1.58)
0.1	4.39 (5.07, 3.81)
0.3	7.74 (8.83, 7.05)
1.0	11.16 (14.33, 9.10)

a) LYMPHOCYTE TRANSFORMATION:

The effect of increasing doses of specific antigen (PPD) on incorporation of tritiated thymidine by tuberculin sensitive guinea-pig lymph node lymphocytes in vitro is shown in Figure 40. The cells at a concentration of 2×10^6 viable lymphocytes/ml were cultured for a total of 96 hours in the presence of antigen and ^{3}H-thymidine was added during the final 24 hours of culture. Antigen caused a dose-related increase of ^{3}H-thymidine uptake by the lymphocytes with an ED_{50} of 8.7 ug/ml for the experiment illustrated.

The effect of E-type prostaglandins on the response to PPD by tuberculin sensitive guinea-pig lymph node cells was tested by adding doses of PGE_1 and E_2 (0.1 and 1.0 ug/ml) to the cultures containing increasing doses of antigen. PGE_1 and E_2 caused a dose-related reduction in the response to increasing doses of specific antigen (Figures 41, 42). At the concentrations used neither PGE_1 nor PGE_2 had a significant effect on lymphocyte transformation in the absence of specific antigen.

b) INCREASED VASCULAR PERMEABILITY:

The effect of E-type prostaglandins on the increased vascular permeability caused by increasing doses of specific antigen in the skin of guinea-pigs sensitised to tuberculin has been examined. Antigen dissolved in saline was injected intradermally in 0.1ml aliquots and ^{125}I-guinea-pig serum albumin in 2.0% Evans blue dye (0.5ml) injected intravenously 20 hours later. Four hours after injection of isotope animals were killed, skinned, the lesions removed and the isotope accumulation measured (see Section 2: Methods viii). PPD (0.2-100 ug/site) caused a dose-related increase in ^{125}I-serum albumin at the site of challenge when injected intradermally into the skin of guinea-pigs sensitised to tuberculin 4 weeks previously (Figure 43). A dose of 20.0 ug/site PPD was used subsequently to test the effect of E-type prostaglandins on the response to antigen.

E-type prostaglandins (0.01-10 ug/site) were injected intradermally into lesions injected with saline or 20 ug/site PPD 20 hours previously. ^{125}I-GPSA was injected intravenously immediately prior to injection of

prostaglandin or saline control and animals were killed four hours following the injection of isotope. In the absence of antigen neither PGE_1 nor PGE_2 caused a significant change in isotope accumulation compared to the effect of saline alone with doses up to 1.0 ug/site (Figures 44, 45). There was, however, a trend towards increased isotope accumulation with increased doses of prostaglandin and doses of 10 ug/site occasionally caused a significant enhancement of isotope levels at the site of injection. At the doses tested (0.01-10 ug/site) both PGE_1 (Figure 44) and PGE_2 (Figure 45) caused a dose related enhancement of isotope accumulation caused by 20 ug/site PPD. The poor effect of PGE's alone at the same doses showed that the observed potentiation of the antigen induced isotope accumulation was not due simply to a summation of the responses.

4) ANALYSIS OF THE POSSIBLE SITE OF ACTION OF E-SERIES PROSTAGLANDINS ON CELLULAR IMMUNE RESPONSES IN VITRO OR IN VIVO

The ability of E-series prostaglandins to affect both in vitro and in vivo cellular immune responses to specific antigen indicates that these mediators of inflammation may play a role in modifying the cellular immune response. Two possible sites of action for such a modification have been examined: (a) an action on the expression of lymphokine activity on specific target cells (e.g. the macrophage in the cell migration test), (b) an action on the generation of lymphokines by T-lymphocytes responding to specific antigen. The first of these possibilities was tested by examining the effect of E-type prostaglandins on the ability of preformed lymphokine to mimic the action of antigen.

5) EFFECTS OF E-SERIES PROSTAGLANDINS ON THE ACTION OF PREFORMED LYMPHOKINE

Lymphokine preparations used in the following experiments were generated by guinea-pig lymph node cells sensitised to bovine gamma globulin and incubated for 24 hours in the presence of 1mg/ml BGG antigen (Section 2: Methods ix). The resulting LK rich supernatants were partially purified preparations similar to those described in Section ii of the Results and caused a dose related increase in peritoneal exudate cell migration inhibition, lymph node lymphocyte transformation and isotope accumulation in the guinea-pig skin. The actual lymphokine preparation used in these

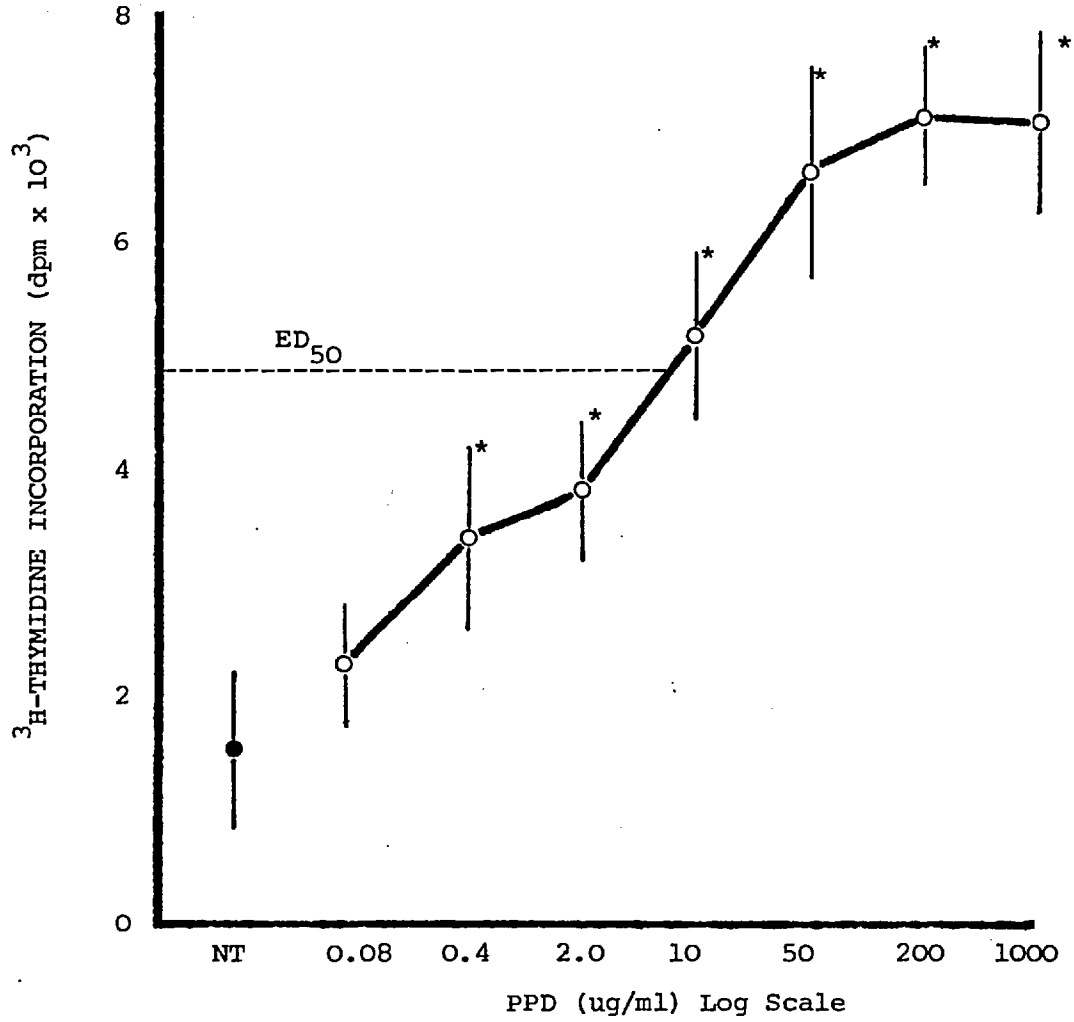

FIGURE 40 EFFECT OF INCREASING DOSES OF SPECIFIC ANTIGEN ON THE INCORPORATION OF 3H-THYMIDINE BY SENSITISED GUINEA-PIG LYMPHOCYTES

Lymph node cells from tuberculin sensitive donor animals were cultured for 96 hours in the presence of increasing doses of tuberculin PPD. ^{3}H-thymidine was added to the cultures 24 hours prior to harvesting. Results are expressed as ^{3}H-thymidine uptake in disintegrations per minute (dpm). Each point represents the mean $\pm$ SD of ten replicate cultures.

ED_{50}: That dose of antigen at which there was 50% of the maximum mitogenic response = 8.8 ug/ml.

●: ^{3}H-thymidine incorporation in the absence of antigen (NT).

o: ^{3}H-thymidine incorporation in the presence of increasing doses of PPD

* Significant enhancement of thymidine uptake. Students t-test $p < 0.05$.

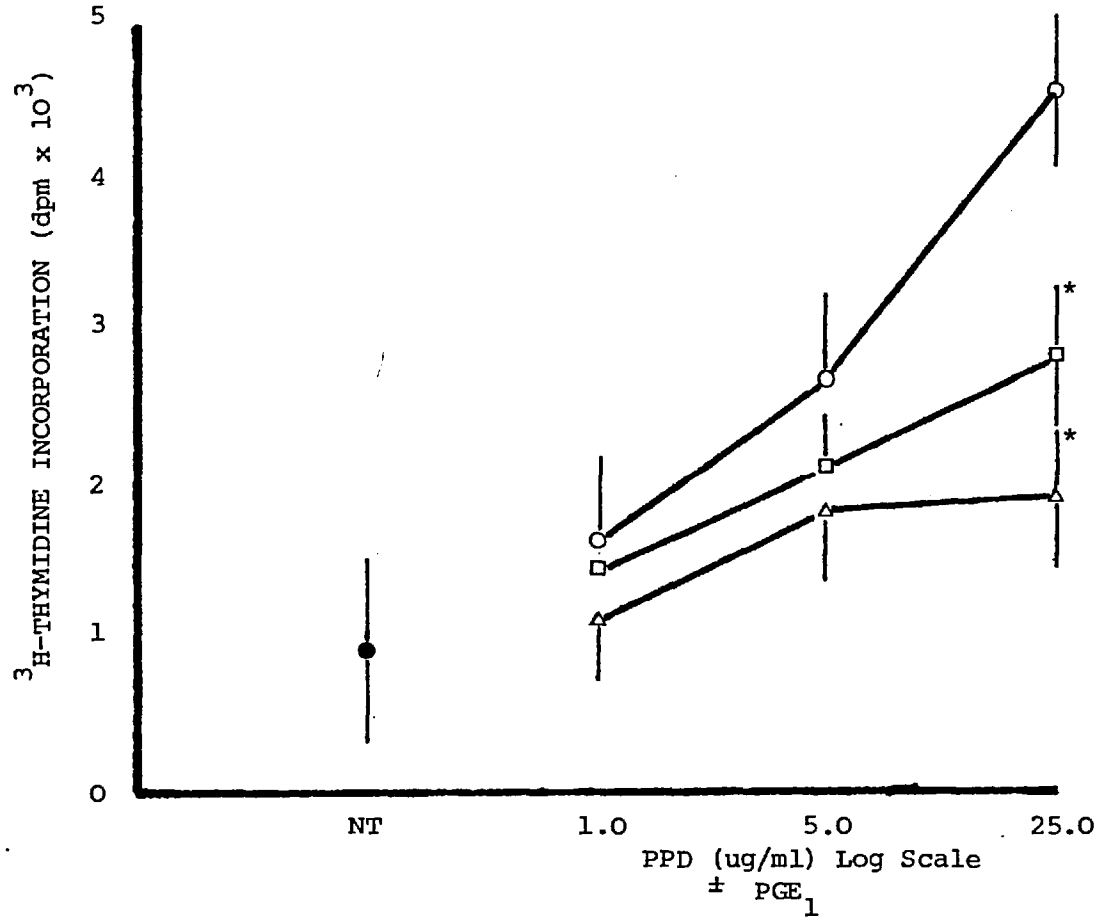

FIGURE 41 EFFECT OF TWO DOSES OF PGE$_1$ ON THE UPTAKE OF THYMIDINE BY SENSITISED LYMPHOCYTES IN RESPONSE TO SPECIFIC ANTIGEN

Lymphocytes from guinea-pigs sensitised to tuberculin were cultured for 96 hours in the presence of increasing doses of tuberculin PPD and in the presence or absence of PGE$_1$. ^{3}H-thymidine was added to the cultures 24 hours prior to harvesting. Results are expressed as dpm. Each point represents the mean ± SD of eight replicate cultures.

● : ^{3}H-thymidine incorporation in the absence of antigen (NT).

○ : ^{3}H-thymidine incorporation in the presence of increasing doses of PPD.

□ : ^{3}H-thymidine incorporation + PPD + 0.1 ug/ml PGE$_1$.

△ : ^{3}H-thymidine incorporation + PPD + 1.0 ug/ml PGE$_1$.

* Significant reduction in the response to antigen. Students t-test $p < 0.05$.

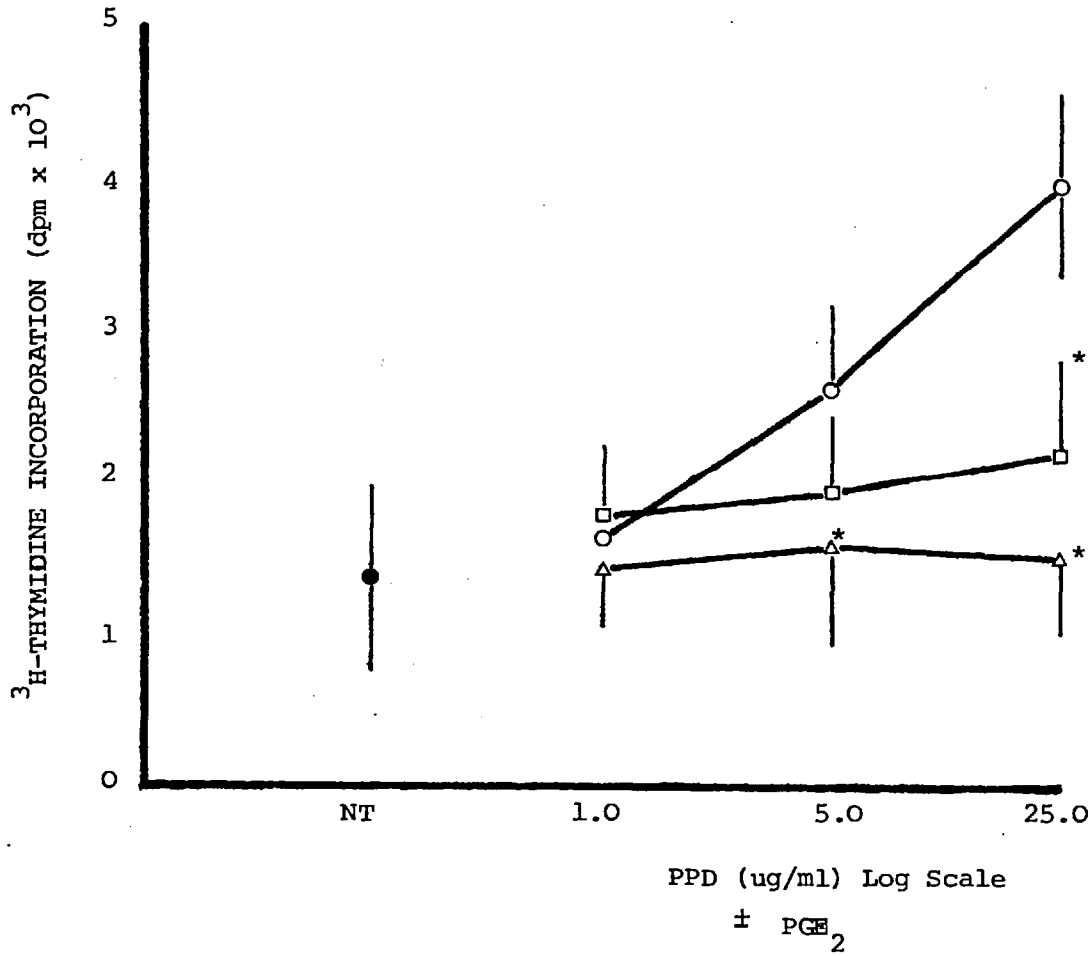

FIGURE 42 EFFECT OF TWO DOSES OF PGE_2 ON THE UPTAKE OF THYMIDINE BY SENSITISED LYMPHOCYTES IN RESPONSE TO SPECIFIC ANTIGEN

Lymphocytes from guinea-pigs sensitised to tuberculin were cultured for 96 hours in the presence of increasing doses of tuberculin PPD and in the presence or absence of PGE_2. ^{3}H-thymidine was added to the cultures 24 hours prior to harvesting. Results are expressed as dpm. Each point represents the mean ± SD of eight replicate cultures.

● : ^{3}H-thymidine incorporation in the absence of antigen (NT).

○ : ^{3}H-thymidine incorporation in the presence of increasing doses of PPD.

□ : ^{3}H-thymidine incorporation + PPD + 0.1 ug/ml PGE_2.

△ : ^{3}H-thymidine incorporation + PPD + 1.0 ug/ml PGE_2.

* Significant reduction in the response to antigen. Students t-test $p < 0.05$.

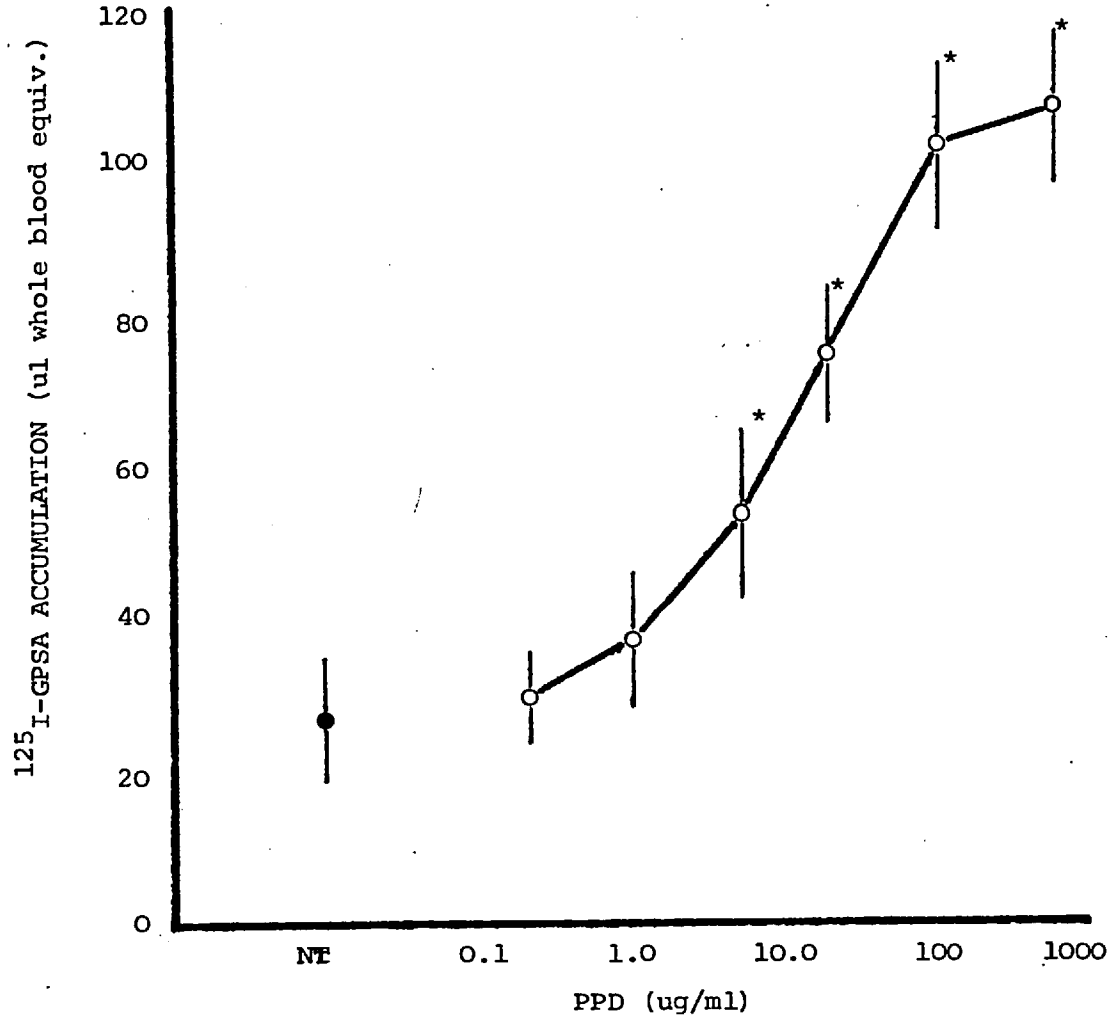

FIGURE 43 EFFECT OF SPECIFIC ANTIGEN ON VASCULAR PERMEABILITY IN GUINEA-PIG SKIN

Recipient tuberculin sensitive guinea-pigs were injected id with increasing doses of tuberculin PPD in saline (0.1ml/injection). After 24 hours ^{125}I-GPSA was injected intravenously and the animals were sacrificed after a further 4 hours. Results were expressed as accumulation of ^{125}I-GPSA in the dermal lesion calculated as ul of whole blood equivalent. Each point represents the mean $\pm$ SD of six replicate injection sites spread over three test animals.

● : ^{125}I-GPSA accumulation following injection of saline (NT).

○ : ^{125}I-GPSA accumulation following injection of increasing doses of PPD

* Significant increase in ^{125}I-GPSA accumulation. Students t-test $p < 0.05$.

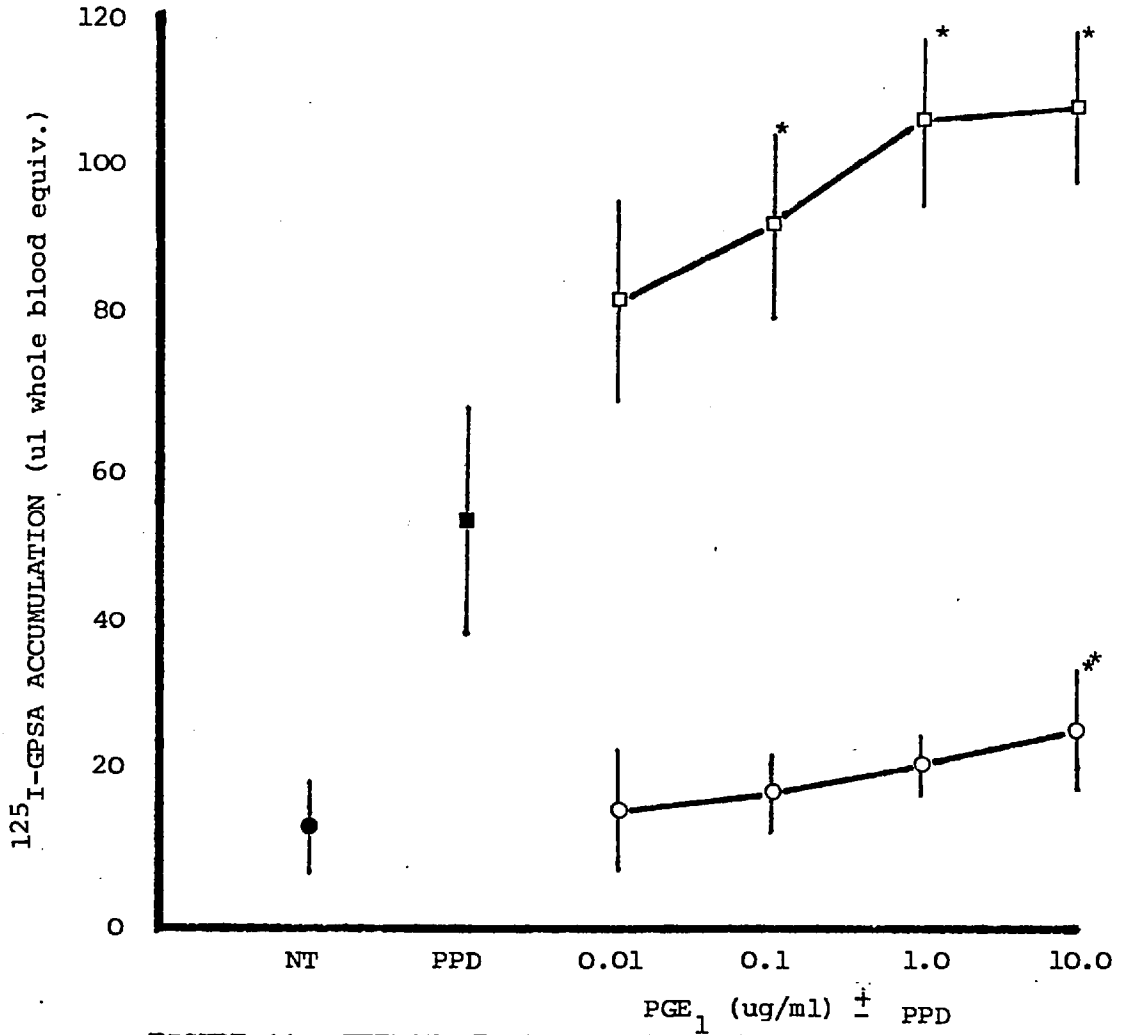

FIGURE 44 EFFECT OF PGE$_1$ ON VASCULAR PERMEABILITY IN THE GUINEA-PIG SKIN IN THE PRESENCE AND ABSENCE OF SPECIFIC ANTIGEN

Recipient tuberculin sensitive guinea-pigs were injected id. with increasing doses of PGE$_1$ ± tuberculin PPD (ED_{50}; 20 ug/ml, see Figure 43). Both drugs and antigen were injected in a total of 0.1 ml saline. After 24 hours ^{125}I-GPSA was injected iv. and animals sacrificed 4 hours later. Results were expressed as ^{125}I-GPSA accumulation calculated as ul whole blood equivalent. Each point represents the mean ± SD of six replicate injections into three animals.

● : ^{125}I-GPSA accumulation following id. injection of saline (NT).

○ : ^{125}I-GPSA accumulation following id. injection of increasing doses of PGE$_1$.

■ : ^{125}I-GPSA accumulation after 20 ug/ml PPD id.

□ : ^{125}I-GPSA accumulation after 20 ug/ml PPD id. + increasing doses of PGE$_1$.

* Significant increase in ^{125}I-GPSA accumulation compared to PPD alone. Students t-test $p < 0.05$.

** Significant increase in ^{125}I-GPSA accumulation compared to saline alone. Students t-test $p < 0.05$.

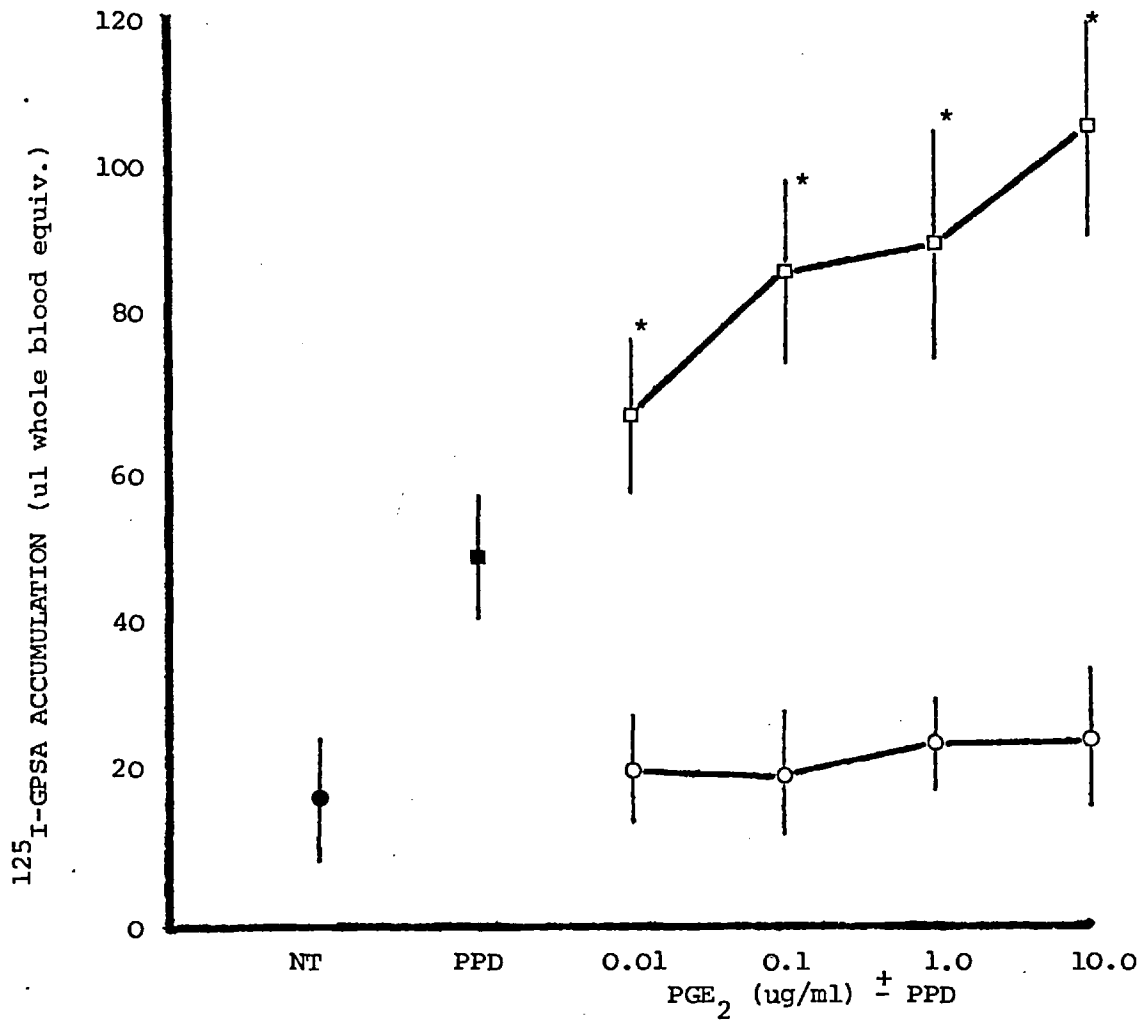

FIGURE 45 EFFECT OF PGE_2 ON VASCULAR PERMEABILITY IN THE GUINEA-PIG SKIN IN THE PRESENCE AND ABSENCE OF SPECIFIC ANTIGEN

Recipient tuberculin sensitive guinea-pigs were injected id. with increasing doses of PGE_2 ± tuberculin PPD (ED_{50}; 20 ug/ml, see Figure 43). Both drugs and antigen were injected in a total of 0.1 ml saline. After 24 hours ^{125}I-GPSA was injected iv. and animals sacrificed 4 hours later. Results were expressed as ^{125}I-GPSA accumulation calculated as ul whole blood equivalent. Each point represents the mean ± SD of six replicate injections into three animals.

● : ^{125}I-GPSA accumulation following id. injection of saline.

○ : ^{125}I-GPSA accumulation following id. injection of increasing doses of PGE_2.

■ : ^{125}I-GPSA accumulation after 20 ug/ml PPD id.

□ : ^{125}I-GPSA accumulation after 20 ug/ml PPD id. + increasing doses of PGE_2.

* Significant increase in ^{125}I-GPSA accumulation compared to PPD alone. Students t-test $p < 0.05$.

studies has been designated as the Kennedy Institute standard LK-1 and the activity of this preparation has been extensively characterised (see section 3: Results 3.ii)a.; Bray et al., 1976).

a) CELL MIGRATION INHIBITION:

Guinea-pig peritoneal exudate cells from unimmunised animals were incubated for 40 hours in the presence of three doses of preformed lymphokine (LK-1) known to lie along the linear portion of the dose response curve (Bray et al., 1976). Two doses of PGE_1 and E_2 (0.1 and 1.0 ug/ml) were added to test cultures and were present throughout the incubation period. Neither PGE_1 nor PGE_2 at the doses examined had any significant effect on the cell migration inhibition caused by the lymphokine (Figure 46).

b) LYMPHOCYTE TRANSFORMATION:

Lymph node lymphocytes from unsensitised animals were cultured at a concentration of 2×10^6 viable cells/ml for 72 hours with doses of LK1 known to lie along the linear portion of the dose-response curve for this preparation (Bray et al., 1976). As with the cell migration assay two doses of PGE_1 and PGE_2 (0.1 and 1.0 ug/ml) were added to test cultures and were present throughout. Neither of the E-series prostaglandins at the doses tested had any significant effect on the uptake of tritiated thymidine by the lymphocytes in response to preformed lymphokine (Figure 47).

c) INCREASED VASCULAR PERMEABILITY:

As with cell migration and lymphocyte transformation the effect of E-type prostaglandins on the response to LK1 was tested. Doses of LK1 (100 and 200ug/site) were injected in 0.1 ml saline into intradermal sites on the dorsal flank skin in unsensitised recipient guinea-pigs immediately following injection of ^{125}I-GPSA. Animals used in these experiments had been pretreated with 1.5mg/kg intravenous mepyramine maleate injected at the same time as the ^{125}I-GPSA in order to eliminate the initial histamine phase of the response to injected protein (Williams & Morley, 1973a; Bray & Morley, 1977). Doses of

PGE_1 or PGE_2 (0.1 and 1.0 ug/site) were mixed with the LK and injected into test sites. Four hours after injection of isotope and drugs animals were killed and the isotope accumulation in injected sites measured. Neither PGE_1 nor PGE_2 at the doses tested had any significant effect on the increased isotope accumulation resulting from injection of the LK (Figure 48).

The doses of prostaglandins used in the above experiments were incapable of affecting the response to preformed lymphokine although they were active in modifying antigen induced cell migration inhibition, lymphocyte transformation and the increased vascular permeability response (Section 3: Results iii) 2, 3). These results demonstrate that the action of E-series prostaglandins on the antigen induced cell mediated immune responses examined does not appear to represent a modification of the ability of lymphokine to act on target cells.

6) EFFECT OF E-SERIES PROSTAGLANDINS ON THE GENERATION OF LYMPHOKINE BY SPECIFICALLY SENSITISED LYMPHOCYTES IN RESPONSE TO ANTIGEN IN VITRO. RESULTANT LYMPHOKINE ACTIVITY MEASURED BY CELL MIGRATION INHIBITION, LYMPHOCYTE TRANSFORMATION AND INCREASED VASCULAR PERMEABILITY

In view of the inability of E-type prostaglandins to modify the effects of preformed lymphokine the second of the possible sites of action for prostaglandin activity mentioned in Section 3: iii) 4 was examined. That is the possibility that E-type prostaglandin may modify the generation of lymphokines by specific T-responding lymphocytes following antigen activation.

In order to test this hypothesis peritoneal exudates derived from animals sensitised to specific antigen were incubated with antigen in the presence of E-type prostaglandins and the resulting lymphokine activity tested using the cell migration inhibition, lymphocyte transformation and increased vascular permeability assays.

Guinea-pigs were sensitised to bovine-gamma globulin (BGG) in Freunds complete adjuvant (FCA) as described in Section 2: Methods ii). Three weeks following immunisation donor animals were skin tested with 10 ug/ml BGG id. and individuals with areas of induration greater than 1.0cm diameter after 24 hours were used. Following induction of PEC donor guinea-pigs

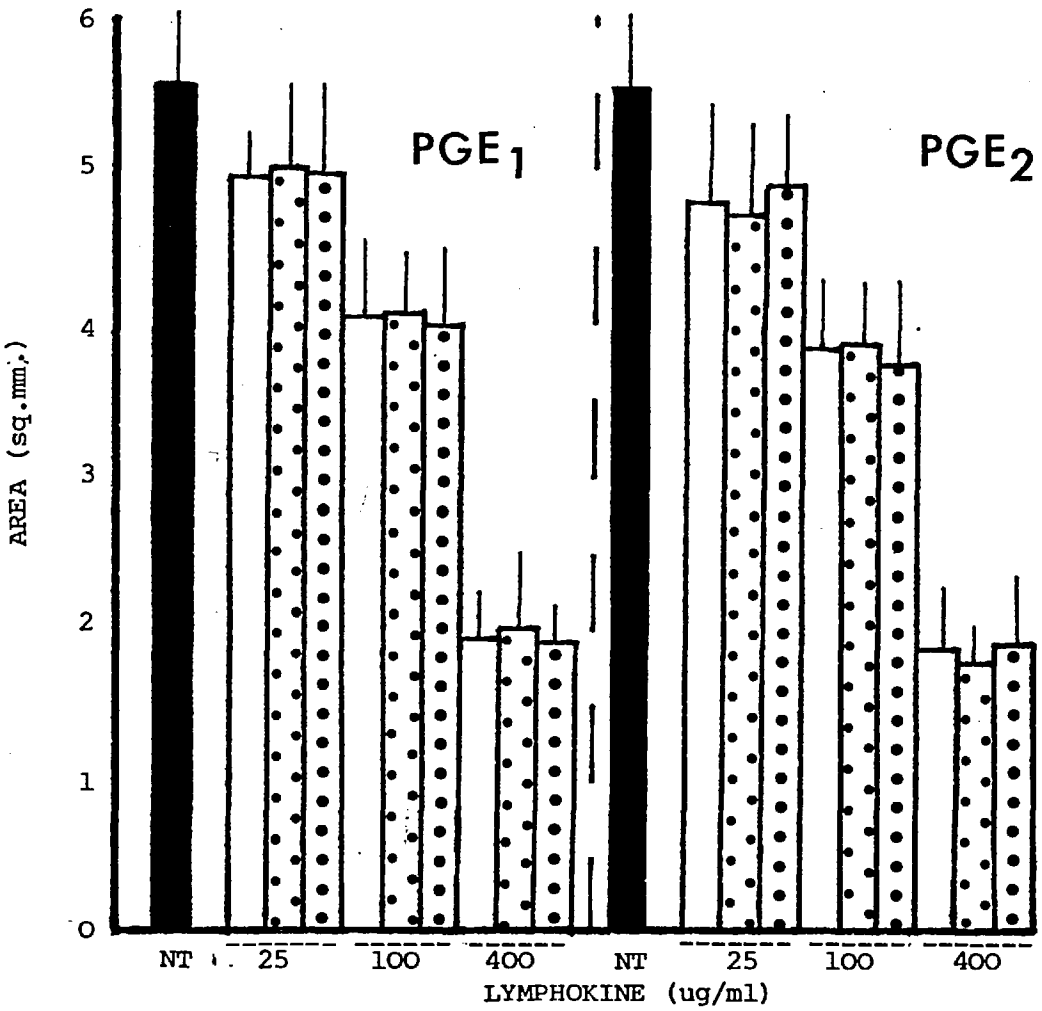

FIGURE 46 HISTOGRAMS ILLUSTRATING THE EFFECT OF TWO DOSES OF PGE_1 AND PGE_2 ON THE GUINEA-PIG CELL MIGRATION INHIBITION RESPONSE TO PREFORMED LYMPHOKINE

PEC from non-sensitised donor animals were cultured for 40 hours in the presence of increasing doses of a freeze dried lymphokine preparation ± two doses of PGE_1 or PGE_2. Results are expressed as area of cell migration in sq.mm. Each bar represents the mean ± SD of eight replicate cultures. There was no significant difference between the treatments in the presence or absence of either dose of PGE_1 or PGE_2. Students t-test $p > 0.2$.

■ Migration in the absence of LK or drug (NT).

□ Migration in the presence of LK alone.

⁘ Migration in the presence of LK + 0.1 ug/ml PGE_1 or PGE_2.

■ Migration in the presence of LK + 1.0 ug/ml PGE_1 or PGE_2.

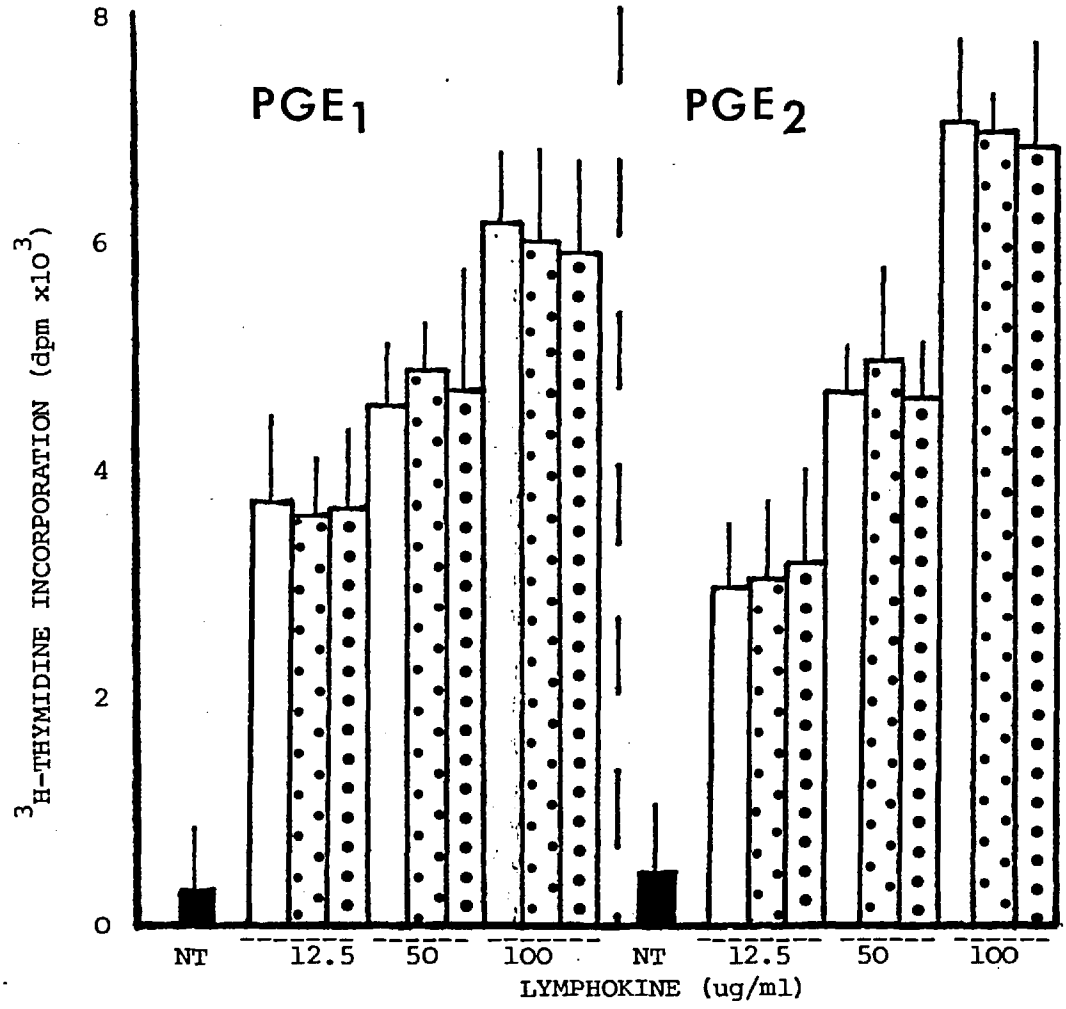

FIGURE 47 HISTOGRAMS ILLUSTRATING THE EFFECT OF TWO DOSES OF PGE_1 OR PGE_2 ON THE GUINEA-PIG LYMPHOCYTE TRANSFORMATION RESPONSE TO PREFORMED LYMPHOKINE

Lymph node cells from non-sensitised donor animals were cultured for three days in the presence of increasing doses of preformed lymphokine $\pm$ two doses of either PGE_1 or PGE_2. ^{3}H-thymidine was added to the cultures 24 hours prior to harvesting. Results were expressed as ^{3}H-thymidine incorporation as dpm. Each bar represents the mean $\pm$ SD of eight replicate cultures. There was no significant difference between the treatments in the presence or absence of either dose of PGE_1 or PGE_2. Students t-test $p > 0.2$.

■ Transformation in the absence of lymphokine or drug (NT).

□ Transformation in the presence of lymphokine.

⚄ Transformation in the presence of LK + 0.1 ug/ml PGE_1 or PGE_2.

■ Transformation in the presence of LK + 1.0 ug/ml PGE_1 or PGE_2.

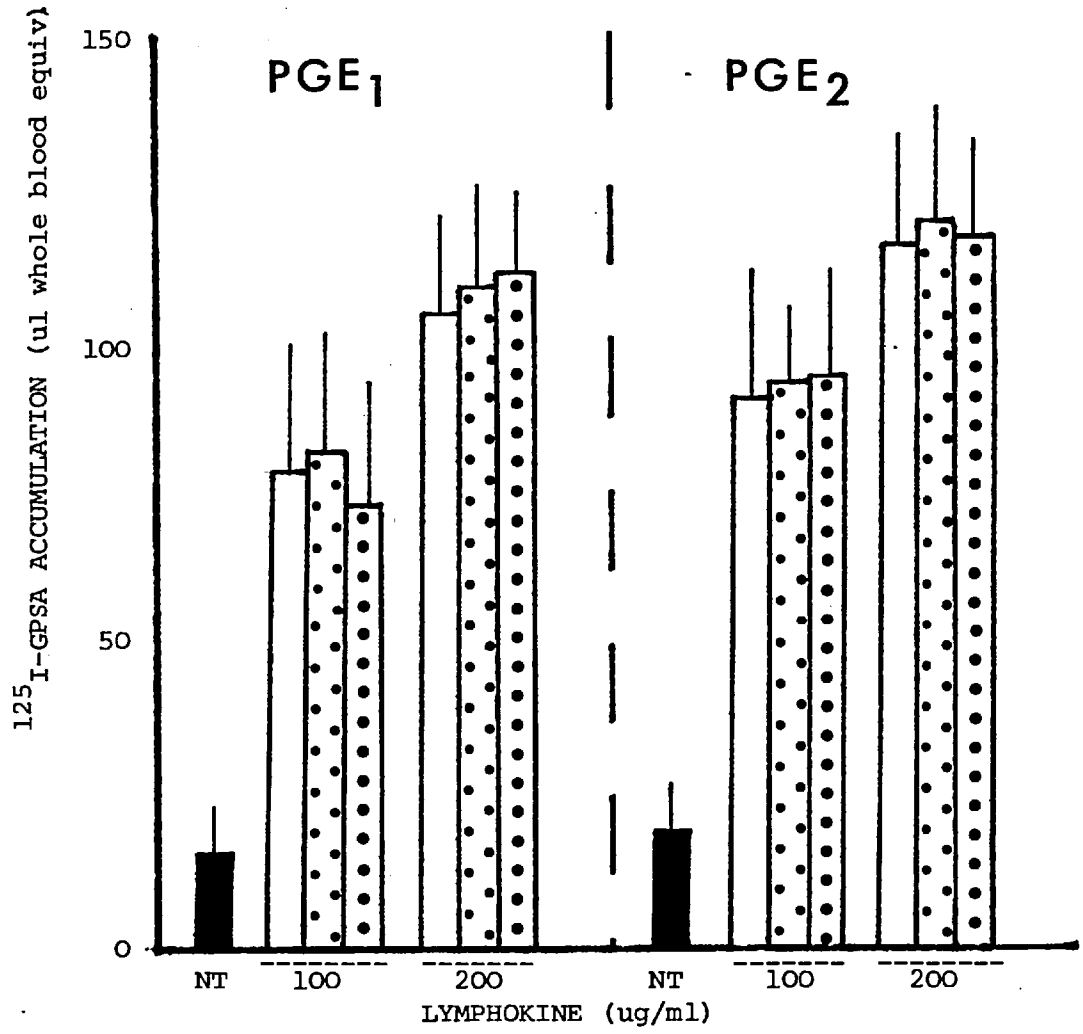

FIGURE 48 HISTOGRAMS ILLUSTRATING THE EFFECT OF TWO DOSES OF PGE_1 OR PGE_2 ON THE INCREASED VASCULAR PERMEABILITY RESPONSES TO PREFORMED LYMPHOKINE IN THE GUINEA-PIG SKIN

Recipient non-sensitised guinea-pigs were injected id. with two doses of preformed lymphokine ± two doses of PGE_1 or PGE_2 four hours prior to sacrifice. Immediately prior to injection of LK animals received 1.5 ug/kg mepyramine maleate and 1-2 uCi ^{125}I-GPSA iv. in 0.5 ml saline. Results were expressed as ^{125}I-GPSA accumulation in ul. whole blood equivalent. Each bar represents the mean ± SD of six replicate skin sites in a total of three animals. There was no significant difference between skin sites treated with either dose of LK alone compared to LK treatment in the presence of PGE_1 or PGE_2. Students t-test $p > 0.2$.

- Saline 0.1 ml (NT)
- Lymphokine alone.
- LK + 0.1 ug/site PGE_1 or PGE_2.
- LK + 1.0 ug/site PGE_1 or PGE_2.

were killed, PEC removed and prepared as described in Section 2: vi a). Falcon plastic culture vessels were prepared containing 1-2 x 10^6 viable PEC/ml in Eagles MEM with 5% heat inactivated normal guinea-pig serum. Each dish contained a total of 20mls cell suspension. Test cultures were incubated in the presence of 1mg/ml BGG in order to generate LK activity. Increasing doses of E-type PG's were also added to test cultures and were present throughout the culture period. The preparations were incubated for 24 hours at 37°C in an atmosphere of 5% CO_2 in air. Following incubation the reaction was stopped and cells removed by centrifugation of the cultures at 400g for 10 minutes at 4°C.

The resulting supernatants were partially purified as described in Section 2: x). High molecular weight material including the inducing antigen BGG and any contaminating immunoglobulin were removed by precipitation at 40% saturation with ammonium sulphate (AS). The supernatants were then made up to 90% AS saturation in order to precipitate the LK rich fraction, the resulting precipitate being redissolved in water and extensively dialysed to remove small molecular weight salts and PG's. Finally, the preparations were freeze dried and stored at -20°C prior to being assayed for LK activity.

Doses of PGE_1 and PGE_2 (0.01 - 1.0 ug/ml) were added to test cultures and the resulting LK preparations tested for their activity in the cell migration, lymphocyte transformation and vascular permeability assays. Cultures incubated in the absence of specific antigen or PG's or with PG's alone were also examined for non-specific LK like activity. Some non-specific LK activity was present in purified culture supernatants from such control cultures, however, there was no significant effect of E-type PG's on this activity and the activity measured was always considerably less than that demonstrated by LK preparations from antigen stimulated cultures.

Lymphokine preparations from antigen stimulated cultures demonstrated substantial activity in all three assay systems tested when compared to controls. The presence of increasing doses of both PGE_1 and PGE_2 during the generation of LK caused a dose-related modification in LK activity recovered from the partially purified supernatants (Figures 49-51). From the data derived from the cell migration inhibition assays doses of PGE_1 from 0.03 - 1.0 ug/ml and PGE_2 from 0.1 - 1.0 ug/ml caused significant (Students t-test: $p < 0.05$) reduction in LK activity, in

addition, detectable, though not statistically significant, effects could be demonstrated with doses of PG as low as 10ng/ml (Figure 49). Addition of E-type PG's similarly inhibited the generation of LK stimulating ^{3}H-thymidine incorporation into lymphocytes with statistically significant (Students t-test: $p < 0.05$) inhibition occurring with doses of PGE_1 and PGE_2 of 0.1 ug/ml and above (Figure 50). Some dose-related inhibition was also evident with lower doses of PG's.

When these PG modified lymphokine preparations were tested for their ability to cause increased vascular permeability in the guinea-pig skin a different result was obtained. A small dose-related increase in the vascular permeability response to LK was observed and this increase was significantly different from the vascular permeability response to LK prepared in the absence of PG's when doses of 0.1 ug/ml ($p < 0.1$) and 1.0 ug/ml (Students t-test: $p < 0.05$) PGE_1 and PGE_2 were incubated with the LK generating lymphocyte populations (Figure 51).

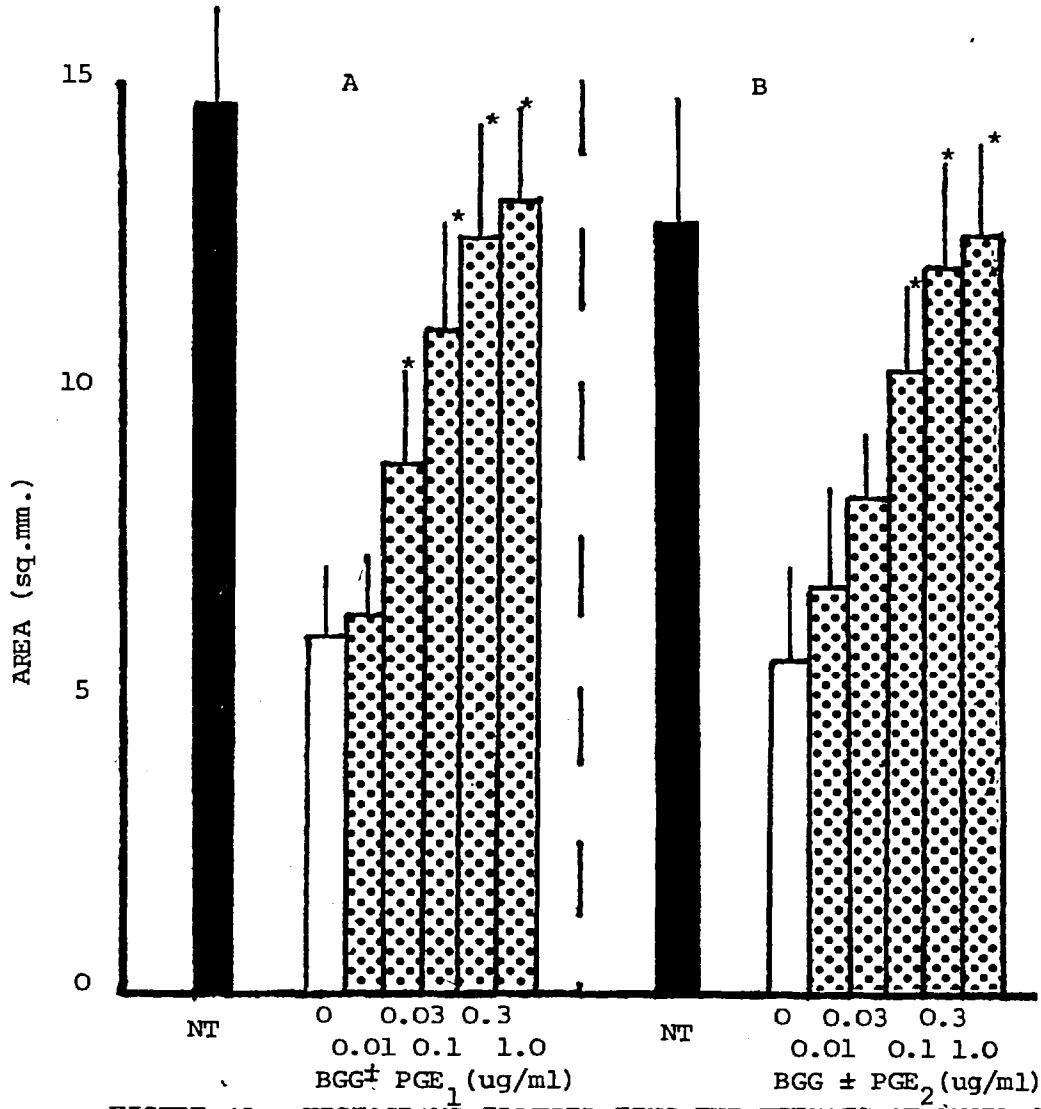

FIGURE 49 HISTOGRAMS ILLUSTRATING THE EFFECTS OF INCREASING DOSES OF (A) PGE_1 AND (B) PGE_2 ON THE GENERATION OF MIGRATION INHIBITORY LK

Guinea-pig PEC from animals sensitised to BGG in FCA was incubated with 1 mg/ml BGG ± PGEs for 24 hours as 37°C. Resulting partially purified lyophilised supernatants were reconstituted and tested for the capacity to cause migration inhibition of normal PEC during 40 hours culture. Results were expressed as area of migration in sq.mm. Each bar represents the mean ± SD of eight replicate cultures.
* Significant reduction in the generation of LK caused by antigen in the absence of PGE. Students t-test $p < 0.05$.

■ Supernatants without antigen or PGE.
□ Supernatants from cultures containing 1 mg/ml BGG.
⊡ Supernatants from cultures containing BGG + increasing doses of PGE.

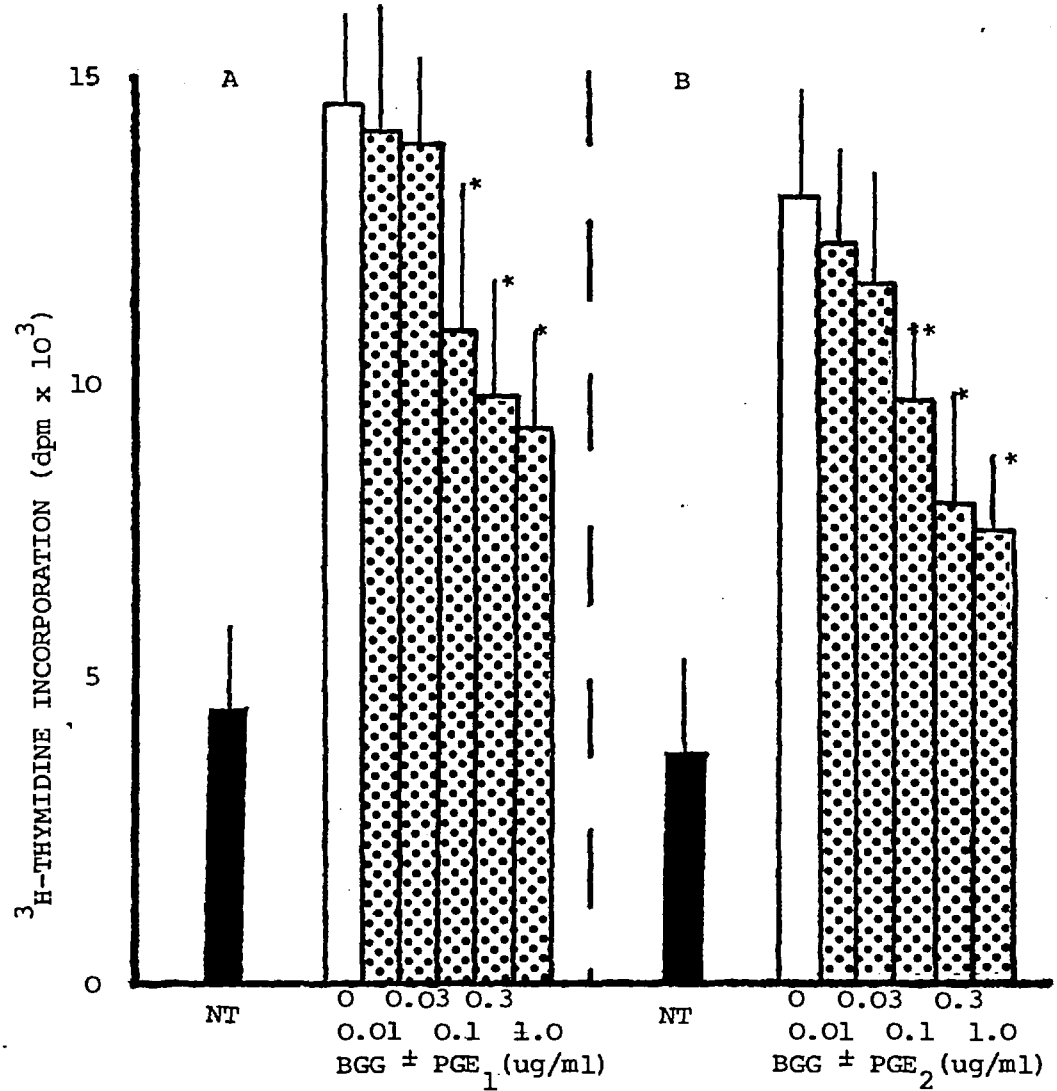

FIGURE 50 HISTOGRAMS ILLUSTRATING THE EFFECT OF (A) PGE_1 AND (B) PGE_2 ON THE GENERATION OF LK CAUSING LYMPHOCYTE TRANSFORMATION

LK rich supernatants were prepared as for Figure 49. Purified supernatants were cultured with lymph-node cells from unsensitised guinea-pigs for 72 hours at 37°C. Results are expressed as ^{3}H-thymidine uptake as dpm. Each bar represents the mean ± SD of eight replicate cultures.

* Significant inhibition of the generation of mitogenic LK. Students t-test $p < 0.05$.

■ Supernatants without antigen or PGE.

□ Supernatants from cultures containing 1 mg/ml BGG.

⁘ Supernatants from cultures containing BGG + increasing doses of PGE.

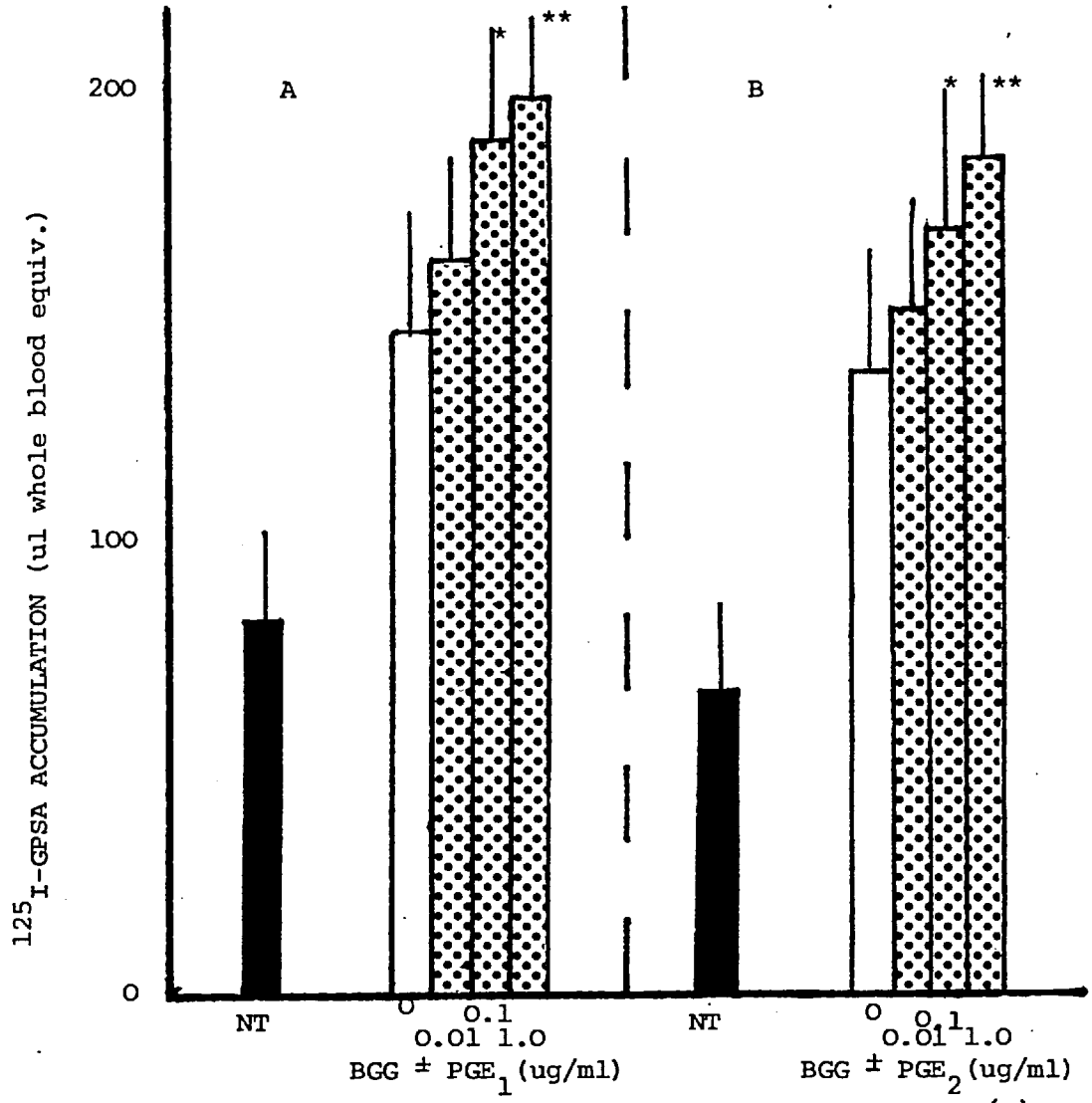

FIGURE 51 HISTOGRAMS ILLUSTRATING THE EFFECTS OF (A) PGE_1 AND (B) PGE_2 ON THE GENERATION OF INFLAMMATORY LK

LK rich supernatants were prepared as in Figure 49. Purified supernatants were injected i.d. into LK-sensitised recipient guinea-pigs 2 hours prior to sacrifice. Results are expressed as ^{125}I-GPSA accumulation in ul whole blood equivalent. Each bar represents the mean ± SD of six sites from three recipient animals.

* Significant enhancement of inflammatory LK generation. Students t-test $p < 0.1$.

** Significant enhancement of inflammatory LK generation. Students t-test $p < 0.05$.

■ Supernatants without antigen or PGE.

□ Supernatants from cultures containing 1 mg/ml BGG.

⊡ Supernatants from cultures containing BGG + increasing doses of PGE.

3 iv) PROSTAGLANDIN GENERATION BY GUINEA-PIG INFLAMMATORY CELL POPULATIONS

In view of the demonstration that E-type PG's could modify the in vitro generation of lymphokines by specifically sensitised lymphocytes responding to antigen stimulation the possibility arises that these hormones may exert an effect on cell mediated immune response in vivo. The corollary to such a proposition is that E-type PG formation should be shown to occur in the vicinity of actively responding T-lymphocytes and should be present in sufficient concentrations to exert an effect on the production of lymphokine.

In order to test whether PG's could be generated by cells involved in the inflammatory response cells from guinea-pig inflammatory peritoneal exudates were examined in vitro for their capacity to generate E-type PG's as measured by radioimmunoassay.

In order to generate inflammatory cell exudates guinea-pigs were injected with 20mls of a 2% sterile starch solution with the animals killed and peritoneal cells removed either 4 hours post injection to recover a neutrophil-rich cell population or three days after injection in order to recover a macrophage-rich exudate. Peritoneal exudate cells from animals that had not received starch were also prepared. Guinea-pig lymph nodes were removed, teased and the resulting lymphocyte-rich cell populations similarly tested for their capacity to generate E-type PG's in vitro. Cells derived from the sources described above were cultured for 24 hours at a concentration of 1×10^6 viable cells/ml in Eagles MEM supplemented with 10% heat decomplemented foetal calf serum at $37^{\circ}C$ in an atmosphere of 5% CO_2 in air (Section 2: Methods xi)). Culture conditions were designed to be essentially the same as those used for the in vitro generation of lymphokine. Following 24 hours of culture the cell suspensions were centrifuged for 10 minutes at 400g and $4^{\circ}C$. Culture medium freed of cells was stored at $-20^{\circ}C$ for up to three weeks prior to assay for E-type PG's using radioimmunoassay (Section 2: Methods xii). In order to ascertain the relative numbers of the different cell types present in the cell cultures slides of cell suspensions before culture were prepared using a cytocentrifuge, fixed in methanol and stained in 1% toluidine blue or geimsa for microscopic examination.

The different cell types obtained from several cultures from each cell source have been tabulated (Table 10). Such cell preparation divides the populations into three major components; neutrophil-rich, lymphocyte-rich or macrophage-rich cell suspensions. After culture the cells were re-examined and viability established by vital dye exclusion.

The prostaglandin productive capacity of the cell populations described above have been examined and the results of several combined experiments presented in Table 11. From these results it appears that all the cell populations studied have the capacity to generate E-type PG activity, however, the inflammatory macrophage rich population generated substantially more activity than either cultures rich in neutrophils or lymphocytes or cultures of PEC derived from guinea-pigs which had not received an inflammatory stimulus. No apparent correlation existed between post culture viability of the cell populations and PG generation as the cells with the lowest initial viability (lymph node cells) and cells with the greatest loss of viability during culture (4 hour PEC) both produced little PG activity (Table 11).

In order to further delineate the inflammatory cell population generating the majority of the PGE-like activity three day starch induced peritoneal exudate cells were cultured for short periods in sterile glass petri dishes and the cells separated into glass adherent and non-adherent fractions. Guinea-pig PEC were incubated at a concentration of 2×10^6 viable cells/ml in Eagles MEM plus 10% heat decomplemented foetal calf serum for one hour at 37°C. After culture the dishes were gently shaken, culture medium containing non-adherent cells removed to new petri dishes and the adherent cells resuspended in medium plus serum. Both adherent and non-adherent cultures were then incubated for a further one hour and the separation procedure repeated. After the second incubation non-adherent cells were washed and cultured at 1×10^6 viable cells/ml for 24 hours in fresh medium plus serum. Adherent cells were removed from the culture dishes by gently scraping with a rubber "policemen", viability was established and the adherent cell population cultured in fresh medium and serum at 1×10^6 viable cells/ml as for non-adherent cells. After culture, supernatant fractions from both sets of cultures were stored

TABLE 10 CELL TYPES PRESENT IN GUINEA-PIG CELL POPULATIONS USED FOR IN VITRO PROSTAGLANDIN GENERATION

Source of Cells	Stimulus	No. of smears*	Cell type (Mean%)				
			Macrophage/ monocytes	Small lymphocytes	Neutrophils	Eosinophils	Basophils
Peritoneal Cavity	None	3	70.6	4.5	4.0	20.1	0.6
Peritoneal Cavity	Starch 2% 4 hours	4	1.6	0.8	94.2	2.6	0.8
Peritoneal Cavity	Starch 2% 3 days	6	74.3	8.7	16.0	1.0	0
Lymph node	None	3	4.2	95.8	0	0	0
Peritoneal Cavity Glass adherent	Starch 2% 3 days	3	89.4	1.0	9.6	0	0
Peritoneal Cavity Glass non-adherent	Starch 2% 3 days	3	34.2	18.6	47.0	0.2	0

* Smears were prepared by placing a drop of cell suspension prior to culture on a slide, air drying the smear, fixing in methanol and staining in 1% toluidine blue for 5 minutes. 200 cells were counted/smear and each smear represents a separate donor guinea-pig

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TABLE 11 PROSTAGLANDIN GENERATION BY GUINEA-PIG CELLS DURING 24 HOUR TISSUE CULTURE

Cell type	No. of Experiments*	Prostaglandin equivalent ng $PGE_2/10^6$ cells/24 hours Mean $\pm$ SEM (Range)	Mean % viability post culture +
Medium alone	3	0.3	-
Unstimulated PEC	4	2.14 $\pm$ 0.16 (0.7 - 3.1)	86
4 hour stimulated PEC	3	0.45 $\pm$ 0.03 (0.2 - 0.62)	81
3 day stimulated PEC	12	11.60 $\pm$ 2.1 (2.4 - 43.2)	94
Lymph node cells	3	1.70 $\pm$ 0.11 (0.2 - 2.8)	61

* Each experiment represents the mean of triplicate cultures

\+ Viability was established by vital dye exclusion (trypan blue 0.4%). Pre-cultured all cells other than lymph node cells were 95% viable. Lymph node cells pre-culture were 70% viable (range 58 - 76%)

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at -20°C and assayed for E-type PG's as described. The separation procedure enriched the adherent fraction in macrophage-like cells and the non-adherent fraction in neutrophils and small lymphocytes (Table 10). After culture the bulk of the PG generating activity as measured by radioimmunoassay was present in the adherent macrophage enriched fraction (Table 12). These results support the proposition that E-type PG production by guinea-pig inflammatory peritoneal exudate cells is due to the macrophage-like glass adherent cell population.

TABLE 12 PROSTAGLANDIN GENERATION BY INFLAMMATORY GUINEA-PIG PERITONEAL CELLS. SEPARATION OF ADHERENT AND NON-ADHERENT CELL POPULATIONS

Cell Type	No. of experiments*	Prostaglandin equivalent ng/PGE_2/10^6 cells/24 hours Mean ± SEM (Range)
Unseparated PEC	3	26.4 ± 3.1 (12.7 - 32.2)
Glass adherent PEC	3	24.2 ± 3.8 (10.8 - 33.1)
Glass non-adherent PEC	3	0.3 ± 0.06 (0.21 - 0.37)

* Each experiment represents the mean of triplicate cultures

3 v) EFFECTS OF ANTIGEN AND LYMPHOKINE ON E-TYPE PG GENERATION BY GUINEA-PIG INFLAMMATORY PERITONEAL EXUDATE CELLS

The demonstration of E-type PG production by adherent macrophage rich guinea-pig PEC coupled with the capacity of PGE to depress lymphocyte generation of some lymphokines suggests that E-type PG's may play a role in a homeostatic regulation of lymphokine secretion (Gordon, Bray & Morley, 1976). Two additional experiments lend support to this proposition. That is the enhanced generation of E-type PG's by guinea-pig PEC in response to specific antigen or preformed lymphokine.

a. ANTIGEN

Macrophage rich PEC were obtained from 3 day starch induced peritoneal exudates from guinea-pigs previously immunised to bovine gamma globulin in FCA (Section 2: Methods ii). Cultures containing 1 x 10^6 viable PEC/ml in 0.5ml Eagles MEM plus 10% heat inactivated foetal calf serum were prepared and increasing doses of BGG added prior to culture. After 24 hours of culture at 37°C in 5% CO_2 in air the cells were removed and the supernatant fluid assayed for E-type PG activity. Doses of specific antigen from 0.1 up to 100 ug/ml caused a significant (Students t-test $p < 0.05$) increase in PGE generation by PEC as measured by radioimmunoassay (Figure 52). Cultures of guinea-pig PEC prepared as above but from animals not previously immunised to BGG did not generate increased E-type PG in response to BGG (10 and 100 ug/ml) as shown in Figure 52.

b. LYMPHOKINE

Macrophage rich guinea-pig PEC prepared as above but derived from unsensitised guinea-pigs were cultured at 1 x 10^6 viable cells/ml as above. Increasing doses of preformed ammonium sulphate purified lymphokine (4.0 - 500 ug/ml) were added to the cultures prior to incubation and were present throughout the 24 hour culture period. After culture the cells were removed and the supernatant fluid assayed for PGE activity by radioimmunoassay. Doses of LK above 20.0 ug/ml caused a significant (Students t-test: $p < 0.05$) dose-related increase in PGE_2 like activity (Figure 53).

Both antigen and lymphokine stimulated cultures were terminated after 24 hours as the E-type PG content of the media did not significantly increase after this time (Bray, Gordon & Morley, 1978).

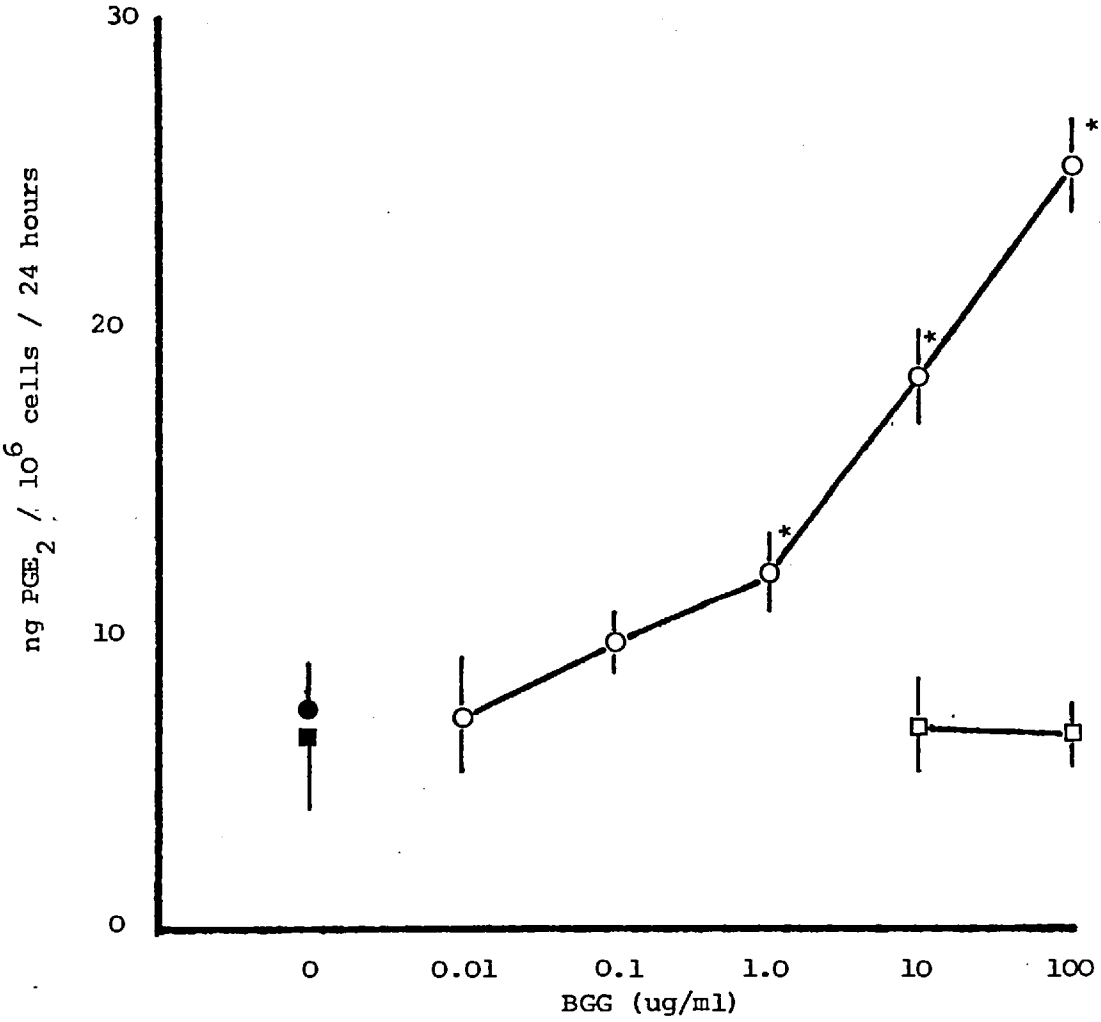

FIGURE 52 EFFECT OF INCREASING DOSES OF SPECIFIC ANTIGEN ON THE GENERATION OF PGE BY INFLAMMATORY GUINEA-PIG PEC

Induced PEC from normal or guinea-pigs immunised against BGG/FCA were cultured at a concentration of 1 x 10^6 viable cells/ml in the presence or absence of increasing doses of BGG for 24 hours at 37°C. Resulting unpurified supernatants were subjected to radioimmunoassay for PGE_2 activity. Results are expressed as ng PGE_2 equivalent/10^6 cells/24 hours. Each point represents the mean ± SD of ten replicate cultures.

* Significant PGE generation over control values. Students t-test $p < 0.05$.

● : Sensitised PEC without antigen.

○ : Sensitised PEC with increasing doses of BGG.

■ : Unsensitised PEC without antigen.

□ : Unsensitised PEC with 10 and 100 ug/ml BGG.

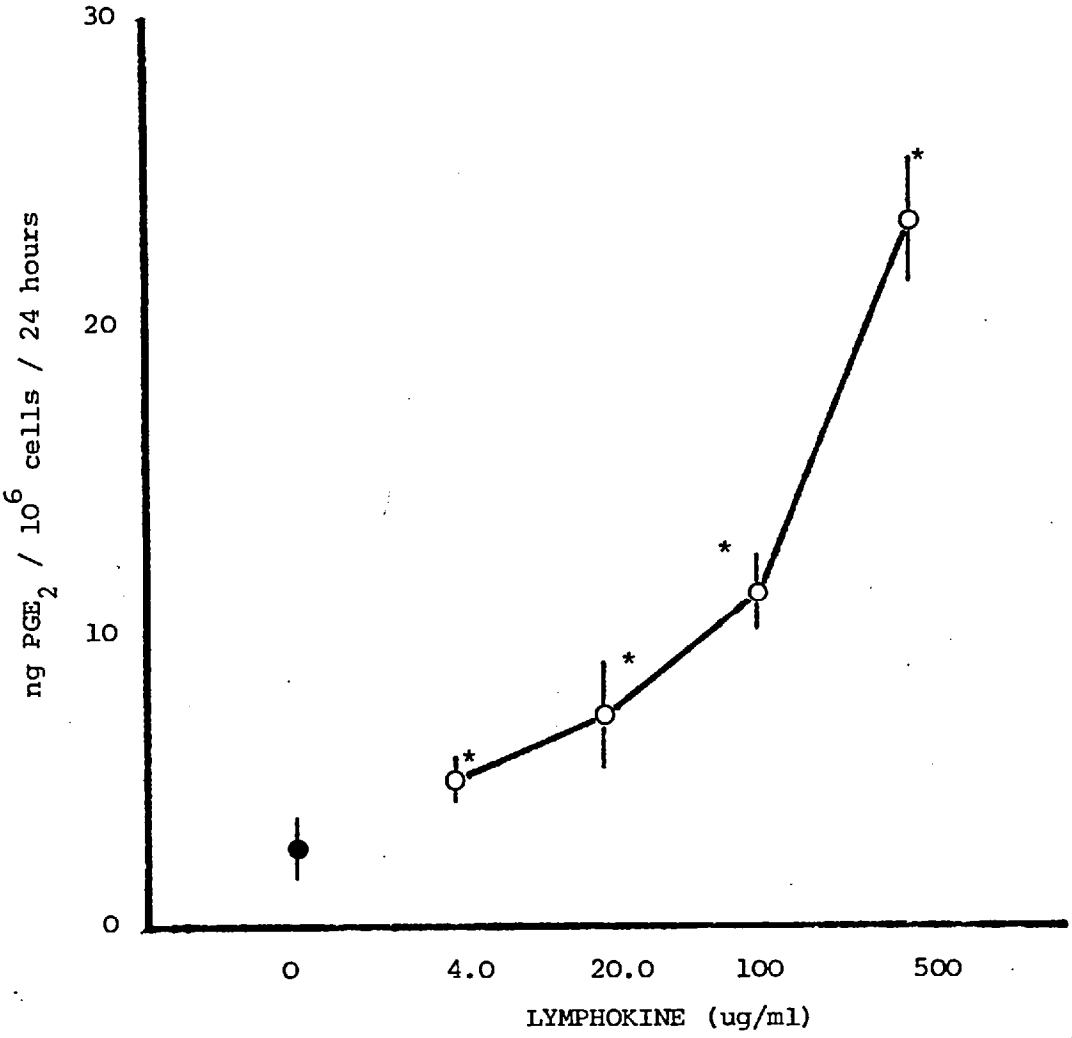

FIGURE 53 EFFECT OF PREFORMED LK ON THE GENERATION OF PGE BY INFLAMMATORY GUINEA-PIG PEC

Induced PEC from normal guinea-pigs was cultured at a concentration of 1 x 10^6 viable cells/ml in the presence or absence of increasing doses of preformed LK for 24 hours at 37°C. Results are expressed as ng $PGE_2/10^6$ cells/24 hours. Each point represents the mean ± SD of ten replicate cultures.

* Significant enhancement of PGE_2 generation. Students t-test $p < 0.05$.

●: PGE_2 generation in the absence of LK.

○: PGE_2 generation in the presence of increasing doses of LK.

3 vi) EFFECT OF INDOMETHACIN ON THE CELL MIGRATION RESPONSES

A third type of experiment implicating E-type PG's as physiological regulators of lymphokine secretion stems from the use of indomethacin, a potent prostaglandin synthetase inhibitor (Flower, 1974). Indomethacin was tested for its ability to modify the in vitro cell mediated immune response of cell migration inhibition in the presence of specific antigen or preformed lymphokine. Indomethacin was also tested for its ability to modify lymphokine generation by specifically sensitised guinea-pig lymphocytes in response to antigen in vitro.

a. EFFECT OF INDOMETHACIN ON CELL MIGRATION INHIBITION

Indomethacin at doses of 0.1 and 1.0 ug/ml did not effect the emigration of guinea-pig inflammatory PEC in the absence of inhibitory antigen or lymphokine (Figure 54). The cell migration inhibition response to 5.0 ug/ml PPD of guinea-pig PEC from animals previously sensitised to tuberculin was markedly enhanced by the addition of increasing doses of indomethacin (0.01 - 1.0 ug/ml). The drug was present throughout the culture period. Doses of drug of 0.1 and 1.0 ug/ml caused a statistically significant enhancement (Students t-test: $p < 0.001$) of the inhibitory response due to antigen (Figure 54). Cells from animals not previously sensitised to antigen were inhibited from migrating in the presence of 100 ug/ml preformed ammonium sulphate purified lymphokine. This response to lymphokine was unaffected by the presence of doses of indomethacin up to 1.0 ug/ml present in the culture medium (Figure 54).

These results indicate that there is some endogenous prostaglandin production by the inflammatory PEC during culture and that the presence of indomethacin inhibits the generation of endogenous E-type PG's and results in enhanced lymphokine formation with a consequent enhancement of cell migration inhibition in response to antigen.

This conclusion has been further tested by determining the effect of indomethacin on lymphokine production.

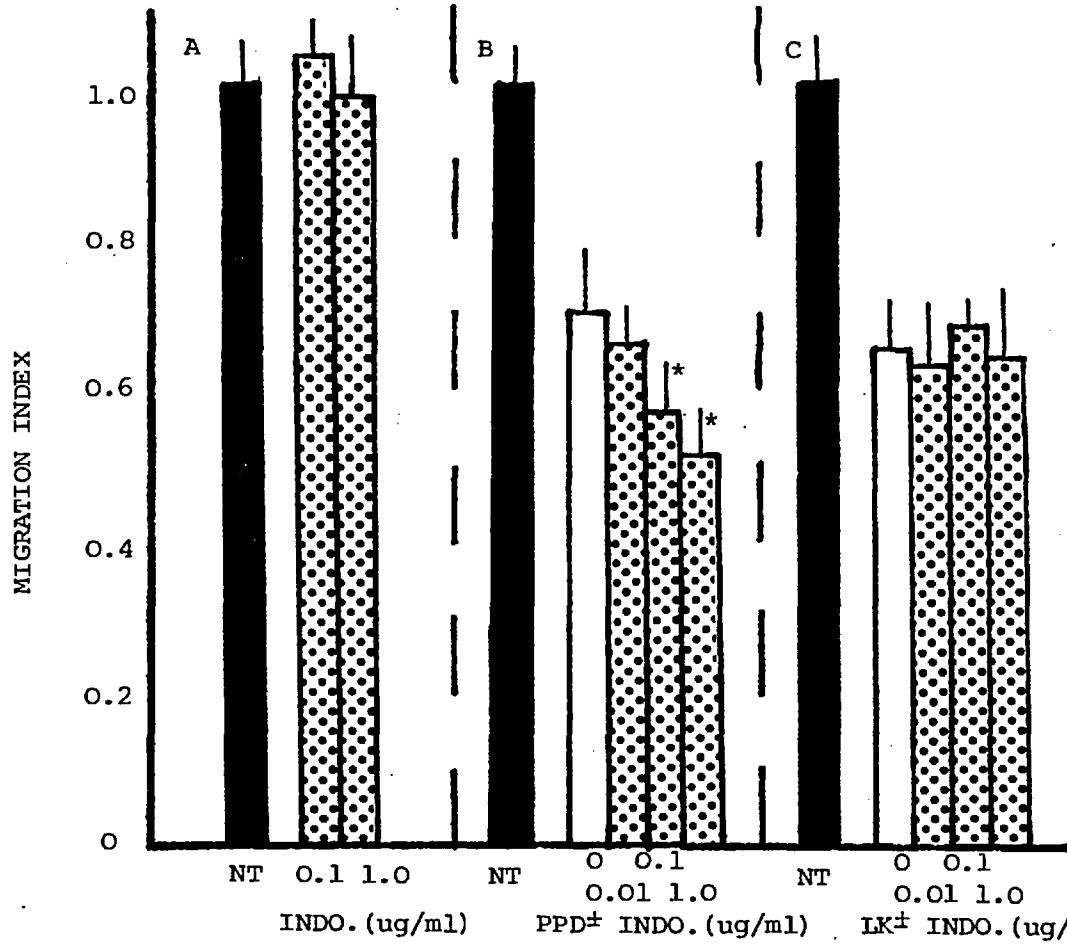

FIGURE 54 HISTOGRAMS ILLUSTRATING THE EFFECT OF INDOMETHACIN ON GUINEA-PIG CELL MIGRATION IN (A) THE ABSENCE OR (B) THE PRESENCE OF ANTIGEN OR (C) LK

Guinea-pig PEC from normal or tuberculin sensitised animals were cultured for 40 hours in the presence or absence of specific antigen (PPD 5.0 ug/ml) or preformed LK (100 ug/ml) and increasing doses of indomethacin. Results are expressed as a Migration Index. Each bar represents the mean ± SEM of six replicate cultures.

* Significant enhancement of antigen induced cell migration inhibition. Students t-test $p < 0.05$.

■ Migrations in the absence of inhibitor or indomethacin (NT).

□ Migration in the presence of antigen or LK.

⠿ Migration in the presence of increasing doses of indomethacin ± inhibitor.

b. EFFECT OF INDOMETHACIN ON LYMPHOKINE GENERATION BY SPECIFICALLY SENSITISED GUINEA-PIG LYMPH NODE CELLS. LK PRODUCTION MEASURED BY CELL MIGRATION INHIBITION

Lymph node cells from guinea-pigs sensitised three weeks previously with BGG:FCA were cultured for 24 hours at a concentration of 1×10^6 viable cells/ml in Eagles MEM plus 10% foetal calf serum in the presence or absence of 1mg/ml BGG and doses of 0.01, 0.1 and 1.0 ug/ml indomethacin. Following 24 hour culture at 37°C the cells were removed and the supernatant medium collected and partially purified via 40% AS fractionation, dialysis and freeze drying (Section 2: Methods x). The resulting LK rich preparations were redissolved in medium and tested for their ability to cause cell migration inhibition. Lymphokine preparations in the presence of BGG alone caused marked inhibition of cell migration compared to preparations derived from cultures incubated in the absence of antigen (Figure 55). The presence of increasing doses of indomethacin resulted in a modest dose-related enhancement of LK activity (Figure 55: 0.1 and 1.0 ug/ml indomethacin; Students t-test: $p < 0.1$); this enhancement was not as marked as that observed when indomethacin was added to cultures of inflammatory PEC responding to antigen.

These results confirmed that the action of indomethacin was to enhance the secretion of lymphokine and further indicated that the source of endogenous E-type PG was unlikely to be the lymphocytes.

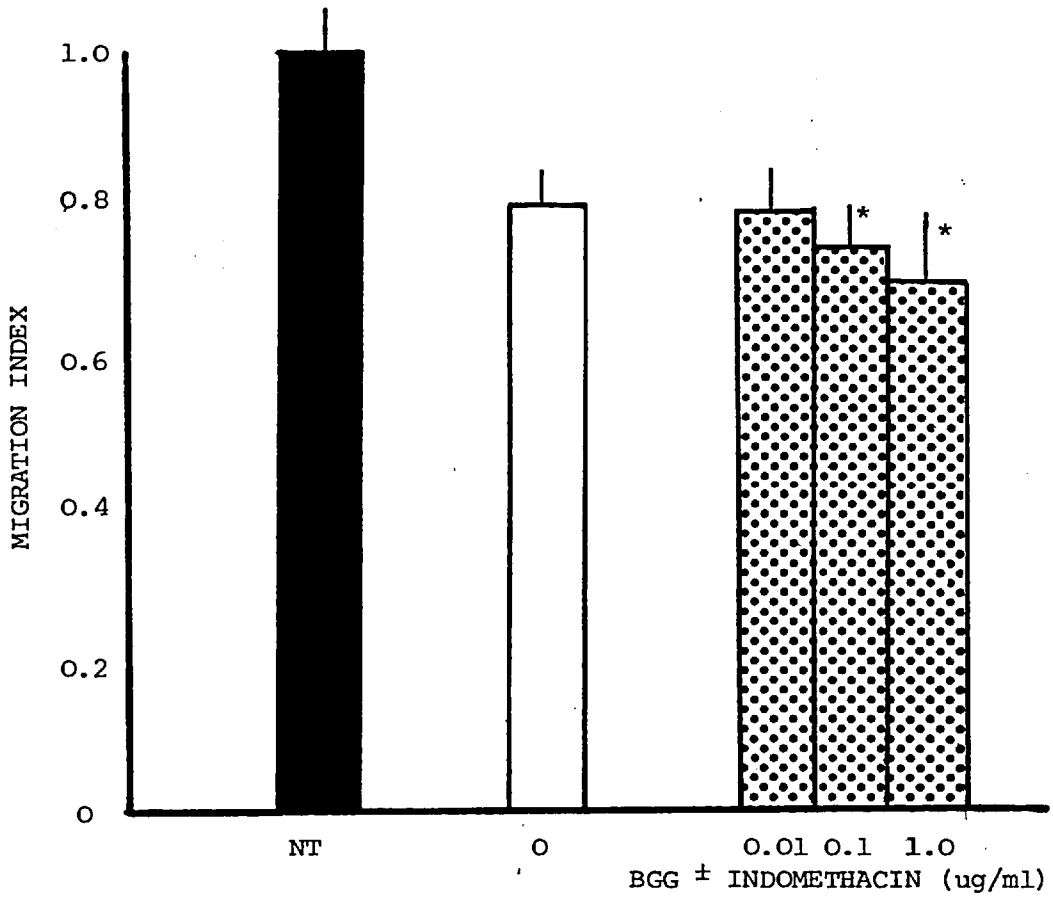

FIGURE 55 EFFECT OF INDOMETHACIN ON THE GENERATION OF MIGRATION INHIBITORY LK ACTIVITY

Lymph-node cells from guinea-pigs sensitised to BGG/FCA were cultured for 24 hours in the presence of 1 mg/ml BGG ± increasing doses of indomethacin. Resulting partially purified lyophilised supernatants were cultured with normal PEC for 40 hours at 37°C. Results are expressed as a Migration Index. Each point represents the mean ± SEM of eight replicate cultures.

* Enhancement of LK generation. Students t-test $p < 0.1$.

■ Supernatants without antigen or indomethacin (NT).

□ Supernatants from cultures containing 1mg/ml BGG.

⁘ Supernatants from cultures containing BGG + increasing doses of indomethacin.

SECTION 4: DISCUSSION

4 i) CELLULAR COMPOSITION OF THE MIGRATION FAN

It was apparent from the high power photomicrographs of the fan of cells formed after 40 hour culture of guinea-pig PEC that the cells constituted a widely dispersed monolayer (Figure 2a-c) and there were several characteristic features of such cell cultures to be seen. Many of the cells showed the intracellular "bleb" formation (Figure 2(c) P) reported previously (Forteza-Vila, Flad & Hochapfel, 1972; Dvorak, Hammond, Dvorak & Karnovsky, 1972) and the early formation of giant cells (polykaryocytes) was also seen as described by Allison and Davies for active macrophage populations maintained in tissue culture (Allison and Davies, 1974). Such giant cells were first described in 1888 in tuberculous lesions (Metchnikoff, 1892) and polykaryocytes have been described in lymph nodes and granulomatous lesions in patients with diseases such as tuberculosis and sarcoidosis (Pearsall & Weiser, 1970). An increased incidence of polykaryocytes has been noted during culture of synovial fluid cells from patients with active rheumatoid and other forms of arthritis (Panayi, Mackay, Neill, McCormick, Marmion & Duthie, 1974). There did not, however, appear from the present study to be an increased incidence of polykaryocyte formation in cultures containing specific antigen which indicated that such giant cell formation was a result of macrophage activation and culture conditions rather than a reaction to antigen stimulation.

A feature not previously noted in guinea-pig PEC cultures was the clumping of polymorphonuclear cells (Figure 2 (b) P) which occurred both in control and antigen treated cultures and such a phenomenon could indicate positive chemotactic activity between neutrophils in such cultures,or, as has been suggested for human PMN's represent increased adhesion (Lomnitzer, Rabson and Koornhof, 1976). There did not appear to be any preferential migration of one or other of the cell types present in the peritoneal exudate however the isolated clumps of active macrophages observed beyond the borders of the cell fan (Figure 2 (a)) indicated that these cells were capable of further and faster migration than the lymphocytes and neutrophils under the culture conditions used during guinea-pig PEC migration.

The only difference visible under the light microscope between control and MIF treated cultures was that in the latter cultures whilst the cell numbers and distribution were similar to control preparation the cells were packed much closer together and in many cases overlapped. This observation is in agreement with previous work (Dvorak et al., 1972) and allows for the close interdigitation of lymphocytes and macrophages in MIF treated cultures observed by Dvorak and other workers using electron microscopy (Dvorak et al., 1972; Fortez-Vila, et al, 1972).

Human peripheral blood leucocyte migrations had a pattern of cell emigration similar to the guinea-pig cell cultures in that the cell fan was made up of a monolayer of homogenous cells on the chamber surface. Some inhomogeneity was occasionally seen when the outer edge of the cell fan had a preponderance of small dark stained polymorphonuclear cells (Figure 4 (a)). These results are consistent with those of Senyk and Hadley who observed a similar cell pattern and in some agreement with the results of Rosenberg & David who also observed an outer "halo" of polymorphonuclear leucocytes (Senyk & Hadley, 1973; Rosenberg and David, 1970). Such results may be explained by the recent observation that PPD causes a greater degree of human peripheral blood mononuclear rather than PMN leucocyte inhibition (Kowalczyk & Zembala, 1978). It was apparent from the results obtained in this study that little information of value could be gained from a comparison of the cell patterns formed during cell migration studies in the presence or absence of inhibitory antigen. Ultrastructural studies of such cells however, indicate that there are considerable differences between antigen treated and control cell cultures such as a loss of cell surface material from macrophages, considerable interdigitation and evidence of transformation of both lymphocytes and macrophages indicated by the presence of polyribosomes and endoplasmic reticulum (Forteza-Vila et al., 1972; Dvorak et al., 1972).

4 ii) TIME COURSE STUDIES

The results of this study point to several useful conclusions which can be drawn from an analysis of the time course of cell migration inhibition. Firstly, such analysis can help in distinguishing between different agents causing inhibition, secondly, in determining the optimal time for measurement and, thirdly, in elucidating the time course of events following the interactions of antigens with lymphocytes during cell mediated immune reactions in vitro.

Guinea-pig peritoneal exudate cells migrating from a capillary tube showed variable rates of migration with time when measured as the overall area of the fan of migrating cells. The S shaped curve shown in Figure 5 represents the typical migration pattern and the reduction in the linear increase in migration area after approximately 40 hours has several possible explanations, such as increased random movement of the migrating cells as the cell density is reduced or depletion of essential materials from the culture medium. A third possibility is that there is a positive chemotactic attraction reducing the cell dispersion as has been suggested by Hughes (1972).

From Figures 7 - 12 it is apparent that different materials able to cause cell migration inhibition follow different patterns of inhibition with time. These differences were especially marked during the first twenty hours of incubation with antigen only causing noticable inhibition after approximately 6-8 hours (Figure 7). Such a delay in the expression of the inhibitory effect of antigen appeared to be a result of the delay in generation of sufficient MIF activity to cause inhibition. The delay in onset of inhibition could be reduced by preincubation of the sensitised cells with specific antigen (Figure 10) however, there remained a period of up to 4 hours during which time there was no difference between control or preincubated cell populations and this was also the case when non-sensitised cells were incubated with preformed LK (Figure 9). The delay in onset of inhibition with antigen is consistent with reports on the time course of MIF generation, assessed by migration inhibition as being 6-8 hours. (Salvin & Nishio, 1969; Svejcar, Johanovsky and Pekarek, 1967c).

It is of interest to note that PHA (a plant mitogen) which is reported to mimic the actions of antigen in cell mediated immunity showed a different time course of cell migration inhibition, an indication that, whilst

PHA is able to cause lymphokine secretion the ability of this material to cause migration inhibition may also be due to other properties of the mitogen such as stimulating cell aggregation.

Material from supernatants of cell culture lines has been shown to cause cell migration inhibition. These culture supernatants have been derived from a variety of sources including immunoglobulin (Ig) producing human lymphocyte cell lines (Tsuchimoto, Tubergen & Bloom, 1972), human T and B cell lymphocyte lines (Florentin, Bruley & Belpomme, 1975; Yoshida et al., 1976) and E. coli cultures filtrates (Yoshida et al., 1975).

The time course of a similar culture filtrate (ODLA) derived from a human, virus transformed fibroblast cell line showed a different time course of cell migration inhibition (Figure 11), when compared to specific antigen. The inhibition occurred within the first hour of culture and the difference in the time course could indicate that the inhibition was not due only to the presence of MIF or that these inhibitory materials do not represent MIF at all. The possibility that such cell line products do not represent "classical" MIF has also been previously suggested (Yoshida et al., 1976) as the MIF activity derived from B and T cell lymphoid lines could not be removed by pre-treatment with anti-lymphokine anti-serum. Physico-chemical studies of human lymphoid cell line MIF, PHA induced MIF and antigen stimulated MIF demonstrate similar properties (Papageorgiou et al., Sorkin & Glade, 1974), so that whilst the MIF activity derived from such cultures may be similar, the time course and anti-sera studies indicate that other inhibitory materials are also present in the culture supernatants.

The time course of migration inhibition by a proteoglycan derived from guinea-pig splenic Kurloff cells (KPP) (Revell et al., 1972) closely mimiced the effects of specific antigen, (Figure 11), however, the delay in onset of inhibition was greater than that shown by preformed lymphokine indicating that whilst this material was able to mimic the action of specific antigen in this test it was unlikely to be the same material as MIF derived from specifically sensitised lymphocytes and would rather appear to stimulate the generation of MIF by the responding PEC.

The time of measurement of the effect of antigen on cell migration in the guinea-pig appears in the past to have been based upon the times used

by Rich and Lewis to assess the effect of old tuberculin (OT) on explant migration and their estimates were in turn based on the time of maximum skin response to OT (24 hours) and the erroneous conclusion that migration inhibition was due to cell death with most cells dead within 48 hours (Rich and Lewis, 1932). Since that time 24 or 48 hours have been used as optimal times of measurement (Bloom & Glade, 1971) and few attempts have been made to validate such a system. Another simpler explanation for the use of 24 and 48 hours may be that these times are the most convenient for the operator.

The most comprehensive attempt to measure the kinetics of cell migration inhibition was made by Hughes who measured the time course of guinea-pig cell migration inhibition to PPD, however, he only measured the effects at a few fixed times up to 30 hours (Hughes, 1972), and whilst his results confirmed the delay in onset in cell migration inhibition in response to antigen, they did not indicate an optimal time for measurement. Hughes also noted that the effects of antigen were reduced with time, the "escape" phenomenon noted previously with guinea-pig cells (Nathan, Karnovsky & David, 1971) and subsequently for human peripheral blood leucocyte migration inhibition (Brostoff, 1974). The present study has helped to demonstrate optimal times for measurement of both guinea-pig and human cell migration inhibition and has also well illustrated the "escape" phenomenon. Both antigen and lymphokine exhibited some degree of "escape" in the guinea-pig system, however, the degree of escape varied with dose of antigen (Figure 7), with, in this case, 0.5 ug/ml PPD showing a reduction in inhibition after 48 hours compared to the higher doses used where no reduction was seen up to 60 hours. Cells in culture with different preformed lymphokine preparations also showed varying degrees of "escape" with time after 30-40 hours (Figure 8). From the results shown it appeared that the optimal time of measurement for antigen induced migration inhibition was 40 hours and the same or earlier (30-40 hours) was optimal for lymphokine preparations. The "escape" phenomenon was more marked with the human peripheral blood migration system and so the time of measurement becomes more critical and although the time of measurement has generally been set at 18 hours, there appears to be little justification for this in the literature (Søberg and Bendixen 1967; Maini , Roffe, McGrath & Dumonde, 1973). The present study demonstrates that the effect of antigen (tuberculin PPD) appears to diminish very quickly after 16-18 hours incubation (Figures 13, 14) so that, in order to measure

the maximum effect of the antigen, cultures should indeed be measured after no longer than 18 hours incubation. The "escape" phenomenon was not seen when PHA was used as the inhibitory agent (Figure 15) providing evidence that the action of PHA on human peripheral blood leucocytes, as with guinea-pig cells, does not completely mimic the effects of specific antigen although the human MIF activity is similar as assessed by Sephadex gel filtration, heat stability and chymotrypsin sensitivity (Papageorgiou, et al., 1974).

4 iii) MEASUREMENT AND CHARACTERISATION OF LYMPHOKINE ACTIVITY

a. USE OF THE CELL MIGRATION INHIBITION TEST AS A BIOASSAY

The recent application of comparative bioassay techniques (Colqhoun, 1971 ch.13) to the measurement of lymphokine activity has demonstrated that a single lymphokine preparation may contain several LK activities (Bray et al., 1976) and such heterogeneity of activity coupled with the evidence from the previous section that the MIF activity of continuous cell line and other culture supernatants does not entirely mimic the action of "classical" MIF makes attempts at lymphokine purification extremely difficult. The use of standard materials such as PHA, ConA or cell line supernatants have been suggested in order to provide a quantitave assay for MIF activity (Bloom & Glade, 1971; Fenton and Taylor, 1975) however, the use of such standards may be misleading if they do not represent a true MIF activity.

The application of comparative, parallel line bioassay using a standard LK preparation has partially overcome the problems described above. Such assays provide unbiased estimates of potency which allow for quantitave analysis of both MIF activity in an unknown preparation and during purification studies.

Several aliquots of a guinea-pig lymphokine preparation showed little variation in activity when measured in the migration inhibition test (Figure 17). Several aliquots from the same lymphokine preparation were similarly measured in two other assays for lymphokine activity (lymphocyte transformation and increased vascular permeability) and the different aliquots also showed little variation in activity in these other tests (Bray et al., 1976). A single lymphokine preparation prepared in bulk and arbitrarily designated as a standard can therefore be used in repeated parallel bioassay in order to test the activity of a particular preparation which may contain different activities in several bioassay systems without recourse to an unrelated "standard" material such as mitogen.

b. LYMPHOKINE HETEROGENEITY

The heterogeneity of lymphokine activities has long been assumed (David and David, 1972) with the bulk of the evidence being provided by biochemical studies on both the character of the materials and their activity in the presence of inhibitors such as puromycin which inhibits MIF activity suggesting that MIF is a protein (David, 1965). Such studies have been supplemented with further experiments on the responses of lymphokines in the presence of different materials and the use of chi-squared (X^2) analysis of 3 + 3 parallel line bioassay data (Bray et al., 1976).

Biochemical data includes estimates of the molecular weights (MW) of partially purified LK activities on Sephadex (G 100) gels. Guinea-pig MIF has been estimated to have a molecular weight of 50-57,000 daltons (Remold et al., 1970), in contrast to the guinea-pig LK cytotoxic to fibroblast cell cultures which has an estimated MW of 35-55,000. Similarly mitogenic factor (MW = 25,000) can be distinguished from MIF (MW = 60,000) (Ashworth, Eckersley & Ford, 1975). Human LK's have been characterised by such techniques and human MIF (MW 25,000) (Rocklin et al., 1972), can be distinguished from a human cytotoxic LK (MW 80,000) (Granger et al., 1970) and from guinea-pig MIF (Rocklin, Winston & David, 1974). Variations in the electrophoretic mobility of LK preparations on poly-acrylamide gels has also been used to demonstrate possible heterogeneity and in separate studies guinea-pig migration inhibitory activity was recovered from pre-albumin fractions (Remold et al., 1970) whereas the peak of the mitogenic activity was recovered from albumin fractions (Dumonde et al., 1969).

The sensitivity of lymphokine preparations to enzymic treatment has demonstrated differences between LK's, thus guinea-pig MIF activity is reduced in the presence of nuraminidase (Remold & David, 1971) but chemotactic activity is not effected (Ward, Remold & David, 1970).

Several other unrelated pieces of evidence serve to indicate LK heterogeneity. Thus, both T and B cells have been shown to produce LK activity following stimulation using a B cell mitogen measured by cell migration inhibition (Yoshida, Sonozaki & Cohen, 1974) whereas, in a separate study, only T cells have been shown to generate mitogenic activity (Rocklin et al., 1974). In the present study the ability of E-type prostaglandins to reduce

the production of the LK activities causing cell migration inhibition (Figure 55) and lymphocyte transformation (Figure 56) is in contrast with the observed enhancement of that activity causing increased vascular permeability (Figure 57) indicating heterogeneity in response to PG's.

Chi-squared analysis of bioassay data has been used to distinguish histamine H_1 and H_2 receptor activities (Black et al., 1972) and that technique has been adapted to demonstrate heterogeneity of lymphokine activity (Section 3: Results ii) and Bray et al., (1976)). Repeated assay of six guinea-pig lymphokine preparations in terms of their macrophage migration inhibitory, mitogenic and inflammatory activities with reference to a seventh "standard" preparation showed that these preparations demonstrated significant heterogeneity within the three activities tested (Table 6). The results showed that the degree of activity varied considerably from one preparation to another and that high activity in one assay system was not linked with similar activity in the other two tests.

The evidence for lymphokine heterogeneity is widespread and increasing however, the question as to whether these various activities can be produced by a single cell type or whether there is lymphocyte heterogeneity in terms of LK production remains largely unanswered. There has been a recent report that lymphocyte subpopulations separated into groups of different sizes by velocity sedimentation generate migration inhibitory, neutrophil chemotactic, lymphotoxic and mitogenic activities after specific antigen or mitogen stimulation (Doroszczak, Yoshida and Cohen, 1977) such evidence indicates that the generation of some lymphokine activities may be ascribed to different lymphocyte subpopulations which appear to be readily separable on the basis of cell size alone.

At a recent lymphokine conference a variety of evidence was presented demonstrating differences between LK activities from a variety of sources. Thus human LIF appears to represent two molecular species and mouse and guinea-pig lymphokines have been separated and purified using techniques such as double isotope radiolabelling and the generation of antisera coupled with chromatography and isoelectric focussing (2nd International Lymphokine Workshop, 1979).

4 iv) MODIFICATION OF CELL MEDIATED IMMUNITY BY DRUGS

a. HISTAMINE AND GUINEA-PIG CELL MIGRATION

The results of the present study have demonstrated that exogenous histamine has the ability to partially reverse the cell migration inhibition caused by specific antigen. The doses necessary are high (10^{-3} - 10^{-4}M) and the inhibition of the response slight (Figure 27). Histamine had no effect on control migrations (Figure 26) at doses up to 10^{-3}M. Neither the H_1 receptor antagonist, mepyramine (at doses of up to 10^{-4}M) nor the H_2 receptor antagonists burimamide and metiamide (at doses of up to 10^{-4}M) had any significant effect on the effect of histamine at doses of 10^{-4}M or 10^{-3}M. These drugs were also unable to affect control migrations or migration inhibition caused by specific antigen with doses of up to 10^{-4}M. (Figures 26-27). The inability of either H_1 or H_2 histamine antagonists to modify antigen induced cell migration inhibition shows that there is no endogenous histamine activity in vitro and indeed the doses of histamine necessary to modify antigen responses in vitro are likely to be considerably higher than the levels present in inflammatory lesions.

Histamine receptors have been postulated to be present on lymphocytes mediating both humoral and cell mediated immune responses (Melmon et al., 1972: Plaut et al., 1973) and this evidence coupled with studies on the role of cAMP in inflammation and immunity (Bourne et al, 1974) has led to a series of observations on the inhibitory effects of histamine on a variety of inflammatory models. For example, the release of lysosomal hydrolases from polymorphonuclear leucocytes (neutrophils) stimulated by immune complexes, a process thought to be responsible for some features of acute inflammation (Weissman et al., 1971), may be reduced following the addition of histamine to such neutrophil cultures responding to antigen-antibody complexes (Weissman et al., 1974; Hensen, 1972). Histamine secretion by human basophils in response to antigen has also been shown to be inhibited by addition of exogenous histamine in vitro (Bourne et al., 1971), providing for a possible negative feedback role in IgE mediated immediate hypersensitivity reactions. The proliferation of B lymphocytes in response to antigen can be detected in vitro by a haemolytic plaque

assay using mouse splenic lymphocytes (Jerne & Nordin, 1963) and using this technique Melmon and his colleagues demonstrated that several vasoactive amines including histamine were able to inhibit plaque formation (Melmon et al., 1974). Finally, there have been several studies on the effects of histamine in delayed hypersensitivity both in vivo and in vitro. Addition of histamine to tuberculin skin tests in man caused a reduction in the intensity of the subsequent lesion (Pepys, 1953) and there have been reports of elevated histamine levels in contact dermatitis (Zachariae, 1964) and tuberculin (Inderbitzin, 1961) skin lesions. Recently Rocklin has reported that 10^{-3}M histamine added to the antigen was able to reduce the delayed skin test response to specific antigen by 50% after 24 hours (Rocklin, 1976). In a similar series of experiments using the histamine H_2 receptor antagonist metiamide Dale was not able to demonstrate any difference in intensity between tuberculin skin tests in untreated guinea-pigs when compared to animals pre-treated with doses of metiamide up to 125mg/kg given 6 hourly before and during challenge (Dale, 1977). These results imply that although high doses of histamine may modify tuberculin skin tests in vivo such an antagonism is not an important control mechanism as H_2 antagonists do not exacerbate the skin response. In vitro tests of d.h. reactions have also been examined for the effects of histamine. Histamine inhibits the cell mediated cytotoxicity caused by T-lymphocytes (Henney et al., 1972) and the responses have been shown to be inhibited by the addition of the specific H_2 receptor antagonist burimamide (Black et al., 1972) and not by H_1 receptor antagonists such as chlorpheniramine (Plaut et al., 1973). Recently, Rocklin has reported that histamine is able to inhibit the cell migration inhibition of guinea-pig PEC in response to specific antigen (Rocklin, 1976). Doses of 10^{-3}M and 10^{-4}M histamine caused a reduction in the response however the lower dose was only effective if low doses of antigen were used. This action of histamine was reversed by burimamide (10^{-3}M) but not by chlorpheniramine (10^{-3}M) implying that the histamine was acting via an H_2 receptor. Rocklin further demonstrated that histamine had no effect on preformed MIF implying that the drug was acting on the lymphocyte.

The results in this study do not confirm the finding of Rocklin and several other studies cast some doubt on the reported effect of histamine on cellular immune responses. Svejcar and his colleagues examined the activity of histamine on the migration inhibition activity of tuberculin in spleen cell

explant studies and could only detect inhibitory activity at doses of histamine from 50-250 ug/ml, doses which they considered were toxic for the target cells (Svejcar et al., 1967b). The hemolytic plaque cell activity (PFC) of histamine has also been recently re-examined and in this study no inhibitory effects on the response could be detected with doses of histamine up to 10^{-3}M (Berenbaum, 1976). If however, no serum was present in the cultures, histamine (10^{-3}M) was able to cause a slight (30%) inhibition in PFC. Destruction of histamine in the presence of serum histaminases could account for this observation and could also explain the lack of effects observed in the present study although in separate experiments the addition of an antihistaminase semicarbazide had no effect on the responses to histamine indicating that serum histaminases were not a significant problem. It is not clear whether serum is present in the cultures used by Rocklin, however, in the same study 15% normal guinea-pig serum was used in lymphocyte transformation tests in the presence of histamine where Rocklin also reports substantial inhibition (42%) of ^{3}H-thymidine incorporation with a dose of 10^{-4} histamine. Recently Beets has demonstrated that some batches of serum contain considerable histaminase activity and can completely remove histamine from culture media within 1-2 days so that care should be taken in interpreting results that do not take into account the possibility of histamine degredation (Beets, 1978; Beets & Dale, 1979).

In vivo neither H_1 nor H_2 histamine antagonists are able to markedly modify increased vascular permeability in the guinea-pig delayed hypersensitivity skin lesion (Morley personal communication). Basophils and mast-cells have been associated by delayed hypersensivity lesions (Wolf-Jurgenson,1966; Askenase, 1976) however, infiltration of these cells soon gives way to large mononuclear cell-rich infiltrates (Dvorak et al., 1970) so that were local histamine responses to be important in delayed hypersensitivity in vivo their role seems likely to be during the early phase of the response although such a time scale does not rule out a role for this mediator in the control of tissue destruction or recruitment of cells to the site of antigen challenge.

Finally a recent report that human mononuclear cells may generate a lymphokine like factor which stimulates histamine release from basophils provides some evidence for a possible role of histamine of cellular immune responses (Theuson, Speck & Grout, 1977).

4 iv) b GLUCOCORTICOSTEROIDS AND GUINEA-PIG CELL MIGRATION

The immunosupressive properties of corticosteroids have been extensively studied, however, the mechanism of action of these drugs on the immune system has not been clarified. The results of the present study confirm the ability of several corticosteroids to inhibit the migration inhibitory activity caused by specific antigen however, the results also demonstrate that the three glucocorticosteroids studied were able to non-specifically enhance cell migration in vitro in the absence of antigen or lymphokine (Figures 31-33).

All three steroids tested increased the area of cell migration in the absence of inhibitory antigen with prednisolone (Figure 33) and dexamethasone (Figure 32) causing significantly enhanced migration with doses ranging from 10ng - 10.0 ug/ml and hydrocortisone enhancing the response at doses of 1.0 and 10.0 ug/ml. All three drugs reduced the migration inhibition caused by antigen over similar dose ranges (10ng/ml - 10ug/ml for prednisolone and dexamethasone and 100ng - 10ug/ml for hydrocortisone). The results indicate that the reduction in migration inhibition caused by cortisone in vitro may partly be caused by a non-specific enhancement of cell migration and is thus not entirely due to specific inhibition of the cellular response to antigen or lymphokine. Several studies have demonstrated that corticosteroids reduce cell migration inhibition in vitro, however, the results have not been entirely consistent. Hydrocortisone (10ug/ml) has been reported to cause a substantial reduction in the response of tuberculin sensitive guinea-pig inflammatory cells incubated with 100ug/ml PPD in agreement with the present study however, the drug was reported not to affect cell migration in the absence of antigen (Balow & Rosenthal, 1973). These authors also reported that hydrocortisone inhibited the response of non-sensitised peritoneal exudate cells to preformed lymphokine but that the steroid (10ug/ml) had no effect on the formation of MIF by tuberculin sensitised lymphocytes in the presence of PPD. In contrast to the results of Balow and Rosenthal a later report has demonstrated that hydrocortisone (10ug/ml) was able to inhibit MIF production by sensitised PEC in the presence of 10ug/ml tuberculin PPD (Wahl et al., 1975), whilst in agreement with Balow and Rosenthal a recent report has stated that the same concentration of hydrocortisone as used in previous studies (10ug/ml)

caused inhibition of LK induced cell migration inhibition from rabbit spleen fragments but did not modify MIF generation (Pekarek et al., 1976). These last authors also noted that, as with the present study, doses of 100ug/ml hydrocortisone caused some enhancement of the cell migration of cultures in the absence of antigen. A similar in vitro culture system to the spleen explants was used by Heilman in 1945 who cultured rabbit splenic explants and showed an enhancement of the migration of "large wandering cells" in the presence of lower doses of hydrocortisone in contrast to the results of Pekarek and his colleagues but similar to the results obtained in the present study (Heilman, 1945). There have since been other reports that corticosteroids are capable to enhancing macrophage migration in vitro (Heilman, 1969: Leahy & Morgan, 1952), whilst in 1973 Stevenson reported that human monocytes in the presence of hydrocortisone generate a factor which enhanced polymorph migration in vitro providing another possible mechanism for the enhanced cell migration observed in the present study (Stevenson, 1973). In vivo administration of prednisolone (3.5mg/kg daily) has been shown to suppress the in vitro migration inhibition response of animals sensitised to tuberculin (Casey & McAll, 1971); similarly in vivo hydrocortisone and prednisolone have been reported to reduce the levels of circulating human peripheral blood monocytes (Fauci & Dale, 1974: Chai & Gelbert, 1973).

Corticosteroids have been studied in a range of cellular immune systems in vitro and have been reported to inhibit the response of lymphocytes to antigen measured by the cytotoxicity of rat lymphocytes on mouse fibroblast target cells (Cohen, 1971; Stavy et al., 1973), release of lymphotoxin from human lymphocytes (Williams and Granger, 1969) and the synthesis of both DNA (Caron, 1967; Blomgren.1974) and RNA (Ono, Teryama, Takaku & Nakao, 1967) by human peripheral blood lymphocytes in response to PHA. In vivo hydrocortisone however has been reported as having no effect on the subsequent in vitro PHA response (Fauci & Dale, 1974). The chemotactic response to antigen by sensitised guinea-pig neutrophils has been reported to be inhibited by hydrocortisone (Ward, 1966) as has the production of chemotactic LK activity by tuberculin sensitive guinea-pig lymphocytes in the presence of PPD (Wahl et al., 1975). The action of a factor thought to be similar to MIF the macrophage aggregating factor (MAF) was also inhibited by hydrocortisone (10^{-4}M) (Granger, 1974).

The results indicate that cortiosteroids in general exert a suppressive effect on the cellular immune response in vitro, however, the data from this study demonstrates that caution should be observed when assessing the actions of these drugs on the cellular immune responses of lymphocyte/macrophage populations in view of their ability to enhance the migration of guinea-pig PEC in vitro in the absence of any immunological stimulus. The varying results obtained by other workers and the wide range of actions in vivo (Claman,1972) of corticosteroids coupled with the problems of species sensitivity, varying lymphocyte reactivity and non-specific effects of these drugs on aspects of the inflammatory process add to the reasons for treating results obtained with these agents with some caution.

4 iv) c) PROSTAGLANDINS AND RESPONSES OF CELLULAR IMMUNITY IN THE GUINEA-PIG

1) EVIDENCE THAT E-TYPE PROSTAGLANDINS ACT AS ENDOGENOUS MODULATORS OF LYMPHOKINE SECRETION

In the present study the ability of a series of prostaglandins to modify cell mediated immunity in vitro has been studied. The data presented demonstrated that E-series PG's were able to markedly reduce the cell migration inhibition response of sensitised guinea-pig PEC in the presence of specific antigen (Figures 35-37; Table 9). Similarly PGE_1 and PGE_2 reduced the ^3H thymidine incorporation by lymph node cells responding to antigen (Figures 41 and 42) whilst in contrast the presence E-series PG's slightly enhanced the vascular permeability response evoked by intra-dermal antigen in sensitised animals (Figures 43 and 44).

Where preformed lymphokine was used in the above tests the addition of E-type PG's had no effect on the cellular response to the lymphokines (Figures 45-47). These results demonstrated that E-type PG's were able to modify lymphocyte reactivity to antigen rather than the response of the target cell or tissue to the lymphokine generated by lymphocytes following antigen stimulation. Further analysis of the effect of PGE_1 and E_2 on the response of lymphocytes to specific antigen demonstrated that in the case of MIF or MF (mitogenic factor) activity the presence of E-type PG's reduced the generation of these materials (Figures 48 and 49). In contrast E-series PG's were able to enhance the generation of the lymphokine material associated with increased vascular permeability (Figure 50).

If the results summarised above were to have any significance in vivo adequate concentrations of E-type PG's would need to be present at the site of antigen/lymphocyte interaction. That such a proposition was feasible has been demonstrated by the ability of inflammatory cell populations to generate sufficient amounts of PGE_2 during in vitro culture (Section 2: Results, Table 11). Separation of such inflammatory cell populations has shown that it was the macrophage like cells which possessed the major capacity for PG generation. The ability of specific antigen or preformed lymphokine to stimulate PGE_2 generation by inflammatory

macrophage rich peritoneal cell populations has also been demonstrated (Section 2: Results, Figures 51 & 52). Finally, the arachidonic acid cyclooxygenase inhibitor indomethacin was shown to slightly enhance the ability of specific antigen to cause cell migration inhibition and to generate MIF activity implying the presence of endogenous protaglandin in cultures of peritoneal exudate cells producing lymphokine activity (Section 2: Results, Figures 53 & 54).

From the series of results outlined above it is proposed that E-type PG's (probably PGE_2) act as homeostatic regulators of cell mediated immune responses in vivo (Figure 56). Thus antigen activation of T-lymphocytes results in the generation of lymphokine activities which, in turn, stimulate macrophages to produce E-type PG's. Such E-type PG production would then serve to reduce further lymphokine generation providing a self-limiting immune response which would be dependant upon a continued antigenic stimulus for its maintenance (Figure 56), (Bray, Gordon & Morley, 1976; Bray, Gordon and Morley, 1978).

2) EVIDENCE FOR PROSTAGLANDIN MODIFICATION OF IMMUNITY IN VITRO AND IN VIVO

During the 1970's considerable evidence has accummulated that PG's, particularly of the E-series, are capable of modifying lymphocyte responses to antigens or mitogens evoking cellular immune reactions. Thus E-type PG's have been shown to inhibit the transformation of human lymphocytes in response to mitogens (Smith et al., 1971; Berenbaum, Cope and Bundick, 1976; Goodwin, Bankhurst and Messner, 1977) and to enhance inhibition of transformation caused by immunosuppressive agents such as procarbazine (Quagliata, Lawrence & Philips - Quagliata, 1973). The PG effects have been shown to be specific to T-lymphocyte populations (Stockman and Mumford, 1974) and similar inhibition of PHA or antigen induced blastogenesis has been shown using polyunsaturated fatty acid precursors of PG's (Mertin and Hughes, 1975; Mihas, Gibson and Hirschowitz, 1975). A recent report has demonstrated the presence of high affinity binding sites for PGE on human peripheral blood lymphocytes (Goodwin, Wiik, Lewis, Bankhurst and Williams, 1979) and these same workers have shown that the response of human T-cell fractions to PG's in terms of

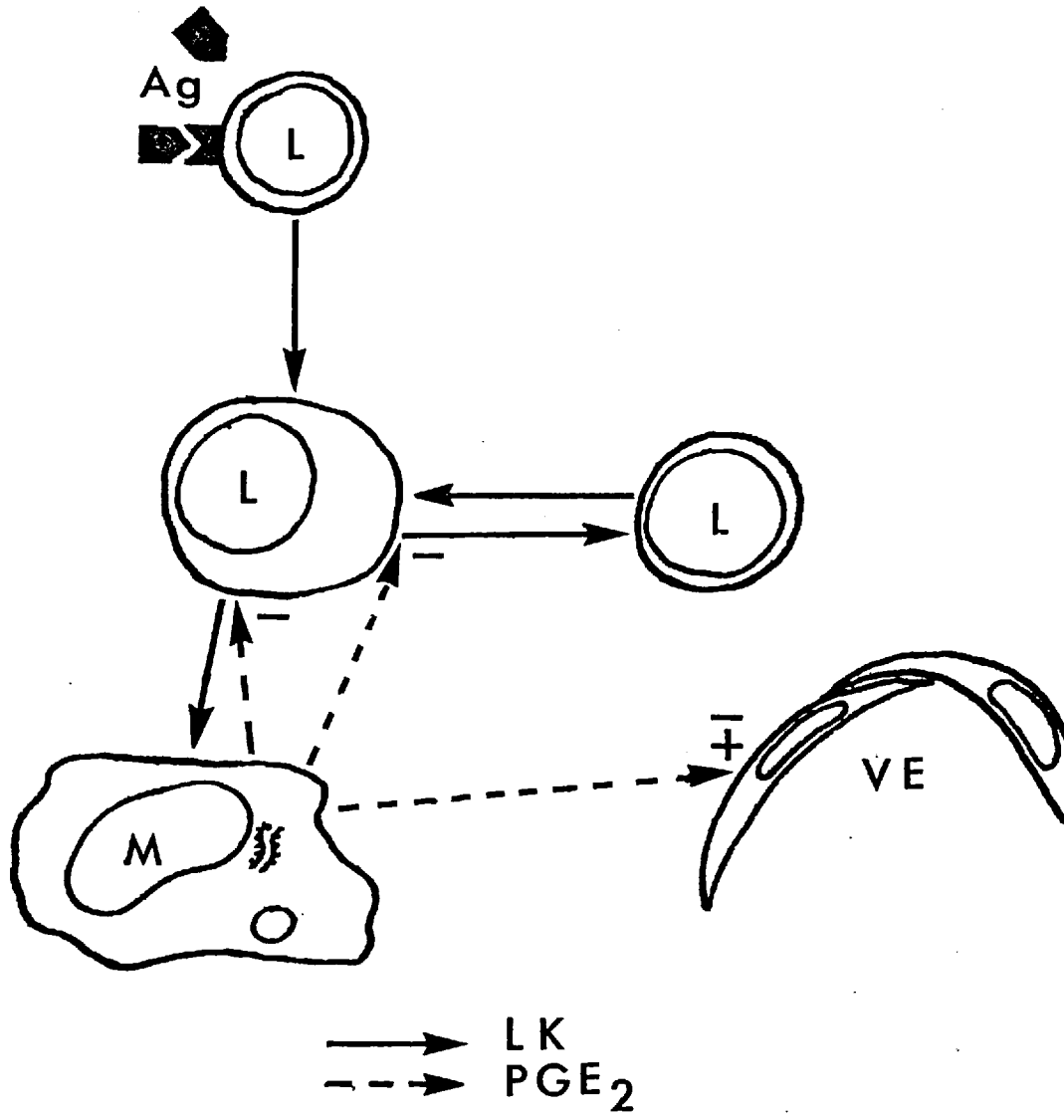

FIGURE 56 PROPOSED NEGATIVE FEEDBACK ROLE FOR PGE_2 ON THE GENERATION OF LYMPHOKINES

Ag: Antigen
L: Lymphocyte
M: Macrophage
VE: Vascular endothelium
LK: Lymphokine

increased cAMP levels varies implying specific subsets of PG-reactive lymphocytes (Goodwin, Kaszubowski and Williams, 1979). The suppression of T-lymphocyte responses has also been examined in terms of lymphokine generation for instance PGE_1 suppresses T-lymphocyte mediated immune cytolysis (Henney et al., 1972) and indomethacin will enhance the generation of guinea-pig macrophage mitogenic factor (Hadden and Englard, 1978). In agreement with the present study Pick and his co-workers have described PGE inhibition of MIF generation (Pick and Manheimer, 1974; Kotkes and Pick, 1975) however, another report has stated that PGE_1 modified the action of MIF on the target macrophage rather than MIF generation by lymphocytes (Koopman, Gillies and David, 1973). This latter report disagrees with the present study and the discrepancy may have arisen due to the large doses of PG (5 and 50 ug/ml) used by Koopman's group; doses which paradoxically may, on their own, cause some inhibition of cell migration (see Section 3: Results, Figure 34). Recently the effects of PG's on human cell migration have been examined and it has been shown that PGE_1 and PGE_2 will inhibit leucocyte migration inhibitory factor generation by human tonsiller lymphocytes (Lomnitzer, Robson and Koornhof, 1976). Another series of reports have implicated PG's in the regulation of antibody secretion in the mouse, thus generation of E-type PG's by murine splenic T-lymphocytes in response to antigen may modulate the generation of 19S antibody both in vivo and in vitro (Webb and Osheroff, 1976). Similarly T-cell regulation of B-lymphocyte responses via PG generation have been proposed (Zimecki and Webb, 1975) and, finally, Webb has suggested that T-cell generation of E-type PG's in response to antigen may modulate the ability of suppressor T-cells to modify responses to antigen (Webb and Nowowiejski, 1978).

The ability of E-type PG's to slightly enhance the in vivo delayed hypersensitivity skin test has been demonstrated (Williams and Morley, 1973b) and the present study provides some explanation for this response as E-type PG's will slightly enhance inflammatory lymphokine generation. However, several reports have shown that non-steroidal anti-inflammatory drugs such as indomethacin which inhibit PG synthesis have little or no effect on delayed hypersensitivity (d.h.) skin lesions (Collier, 1967; Paul, Beets, Bray and Morley, 1978). The observed potentiation of the d.h. lesion noted by Williams and Morley is likely therefore for the most part to reflect the ability of PG's to enhance local blood flow (Williams,

1978) rather than their modest capacity to modify inflammatory lymphokine generation. The ability of PGE's to modify other models of allergic disease could, however, be explained in terms of a modification of LK secretion. For instance, administration of high doses of E-type PG's to rats with adjuvant arthritis reduced the swelling and oedema (Zurier & Quagliata, 1971) and such a response also occured in adrenalectomised rats thus eliminating the possibility of stimulation of steroid release (Zurier et al., 1973). Recently inflammatory models have been developed in animals deprived of essential fatty acids, thus unable to make prostaglandins, and using such a model Bonta and his coworkers have shown an increase in granuloma formation and enhanced collagen biosynthesis following carrageenan injection (Bonta, Parnham and Adolf, 1977; Parnham, Shoshan, Bonta and Neiman-Wollners, 1977). Similar results have been demonstrated following treatment of animals with indomethacin during tuberculin induced pleuresy when enhanced cell accumulation, exudate volume and lysosomal enzyme release were noted (Blackham and Woods, 1979).

3) PROSTAGLANDIN GENERATION BY INFLAMMATORY CELLS

Cellular metabolism of phospholipids leads to the generation of a variety of biologically active fatty acids including the classical prostaglandins such as the PGE's and the PGA's, the recently characterised prostaglandin PGI_2 and the thromboxanes. As well as the above generated following steps involving a cyclooxygenase enzyme a second metabolic pathway via a lipoxygenase leads to a series of hydroxy acids and also the generation of slow-reacting substance of anaphylaxis (SRS-A).

In recent years a variety of cellular elements have been shown to be able to metabolise phospholipids (in particular the precursor of the PG_2 series - arachidonic acid). The ability of the macrophage to generate E-type PG's has been demonstrated by this study and the capacity of such cells to form PG's has been implied in a previous report showing that the macrophage possesses a high proportion of arachidonic acid in membrane phospholipids (Mason, Stossel and Vaughan, 1972). As well as having membrane bound PG precursors macrophages are known to possess the enzymes phospholipase A_1 and A_2 which catabolise phospholipids to form arachidonic

acid and which can be activated during phogocytosis (Munder, Ferber, Modolell and Fischer, 1969; Franson and Waite, 1973), when such cells undergo lipid peroxidation (Stossal, Mason and Smith, 1974). Such evidence confirms that macrophages have the capacity to generate fatty acid metabolites of phospholipids such as the prostaglandins. Since the completion of this study several workers have demonstrated PGE_2 generation by macrophages in the guinea-pig (Glatt, Kalin, Wagner and Brune, 1977), the mouse (Humes, Bonney, Pelus, Dahlgren, Sadowski, Kuehl and Davis, 1977; Brune Glatt, Kalin and Peskar, 1978), and man (Myatt, Gordon, Bray and Morley, 1975; Kurland and Bockman, 1978; Morley, Bray Jones, Nugteren and Van Dorp,.1979). Several of these groups have also been able to demonstrate substantial production of thromboxanes and, in the mouse, significant generation of PGI_2. The nature of the PG generation reported in this thesis has been analysed by gas chromatography and mass fragment analysis and has been confirmed as being PGE_2 (Morley et al., 1979), however, as significant levels of thromboxane B_2 were also detected a possible role for this product of arachidonic acid metabolism in the regulation of lymphokine secretion cannot be excluded although it is inactive in terms of inhibition of mitogen induced lymphocyte transformation (Beets and Gordon, 1979). Generation of PGE_1 has been reported by rat peritoneal macrophages during phagocytosis of materials such as zymosan (Gemsa, Seitz, Till and Resch, 1978). Neutrophils have been shown to generate substantial quantities of PGE_1, E_2 and $F_{2\alpha}$ during phagocytosis (Higgs, McCall and Youlten, 1975; Zurier and Sayadoff, 1975; Tolone, Bonasera, Brai and Tolone, 1977) and human lymphocytes generate small amounts of PGE_2 and $PGF_{2\alpha}$ (Ferraris and de Rubertis, 1974; Webb and Osheroff, 1976). Eosinophils have been demonstrated to generate PGE in response to antigen stimulus and this observation has led to the proposition that these cells may act as modulators of immediate type hypersensitivity responses (Hubscher, 1975).

In a comprehensive examination of a variety of haematogenous cells of both man and guinea-pig carried out in our laboratories only macrophage like cells derived from the guinea-pig peritoneal cavity or human alveoli were capable of generating substantial quantities of PG's during 24 hour culture (Morley et al., 1979). Human peripheral blood monocytes appear to be distinguishable in terms of their PG productive capacity and at least four distinct cell fractions can be shown to differ in their

generation of PGE_2 *in vitro* (Goldyne, Kennedy and Stobo, 1979).

The ability of specific antigen to stimulate leucocyte prostaglandin generation reported in this study has been confirmed using human peripheral blood leucocytes from Ragweed sensitive donors (Okazaki, Burek, Attalah, Reisman and Arbesman, 1975). These authors demonstrated increased PGE and PGF levels following challenge with antigen and further noted that the PG generation was confined to those cell populations able to generate MIF. In view of the present study a likely explanation of the above is that the antigen stimulated T-lymphocyte population was causing monocyte generation of PG's via lymphokine secretion. In a study of murine splenic cells in culture Ferraris and de Rubertis demonstrated increased PGE_2 generation in the presence of mitogen (PHA) or antigen (staphylococal enterotoxin B). These authors were able to partially purify the splenic cell population and concluded that the cells generating the PG activity were present in the non-lymphocyte, glass adherent monocyte population (Ferraris and de Rubertis, 1974). Similarly a PGE_2 generating human peripheral blood, glass adherent monocyte has been described (Goodwin, Bankhurst and Messner, 1977). In addition to demonstrating PG generation by human monocytes these authors were able to show that this cell population would inhibit T-cell (PHA) and to a lesser extent B-cell (PWM) mitogen induced lymphocyte transformation. Recent reports have demonstrated that several lymphokine like materials will stimulate PGE synthesis, thus lymphocyte factors from human gingiva, cartilage and, of particular interest, synovial tissue will stimulate PGE_2 generation from human blood mononuclear cells (Englis, D'Souza, Meats, McGuire, Wright and Russell, 1979) and a human mononuclear cell factor will stimulate adherent synovial cells to generate PGE_2 (Dayer, Goldring, Robinson and Krane, 1979). Such results tend to confirm the proposal put forward in this thesis that macrophage/monocyte prostaglandin generation acts as a homeostatic regulator of T-lymphocyte function. Of particular interest in the context of chronic inflammatory disease are the reports of an adherent macrophage like synovial lining cell capable of generating substantial PGE like activity *in vitro* (Robinson *et al.*, 1973; Dayer *et al.*, 1979). Similar results have been obtained in our laboratory using synovial tissue explants (Sturge, Yates, Gordon, Franco, Paul, Bray and Morley, 1978).

4) OTHER IMPLICATIONS OF MACROPHAGE PROSTAGLANDIN GENERATION

Prostaglandin generation by macrophages could contribute to the heterogeneity of lymphokine activities described in this thesis (see Section 3: Results, ii) and Bray et al., 1976) as the generation of skin inflammatory activity (IF) was not inhibited by E-type prostaglandins whereas production of migration inhibitory and mitogenic activities were reduced (See Section 3: Results, iii c.6).

Other workers have implicated macrophages in the modulation of lymphocyte reactivity to antigen (Gershon, 1974) and factors released from macrophages appear capable of both enhancing and inhibiting lymphocyte proliferation (Waldman and Gottlieb, 1973; Calderon and Unanue, 1975), and in some circumstances the proportion of macrophages in the cultures has been shown to control the response. Thus lower macrophage densities caused enhancement of the lymphocyte response to antigen whilst larger numbers of macrophages (1:1 - 10:1) caused inhibition (Keller, 1975). Similar effects have been reported with E-type PG's with low concentrations causing mouse thymocyte proliferation (Franks, McManus and Whitfield, 1971) and higher concentrations causing inhibition both of human and animal lymphocyte transformation in response to both mitogens and antigens (Gordon et al., 1976; Smith et al., 1971). Ranney has proposed that low molecular weight factors released from lymphoid tissue which inhibit lymphocyte action may be generated by macrophages and biochemical characterisation has led to the supposition that one such factor may be PGE_1 (Ranney, 1975) although from the results of the present study the material appears more likely to be PGE_2. It would seem reasonable to consider that PGE_2 could contribute to the inhibition of lymphocyte activation by low molecular weight factors released by macrophages and thought to play a part in antigen processing (Calderon and Unanue, 1975). In the context of prostaglandin modulation of lymphocyte reactivity and lymphokine generation an interesting series of papers has demonstrated that a dialisable product of foetal human monocytes could inhibit lymphocyte proliferation and leucocyte migration inhibitory factor generation (Wolf , Lomnitzer and Rabson, 1977; Wilson and Remington, 1979). The possibility exists that such material could be PGE_2 and, if so, would implicate PG's in the depressed cell mediated immune responses seen during pregnancy (Wolf et al., 1977).

The ability of E-type PG's to enhance levels of cyclic 3-5 adenosine

monophosphate (cAMP) in leucocytes (Bourne et al., 1974) and macrophages (Remold - O'Donnell, 1974; Gemsa, Steggemann, Menzel and Till, 1975) coupled with the evidence that cAMP inhibits the release of lysozomal enzymes (Weissman et al., 1971) provides a mechanism for the autoregulation of inflammatory macrophage activity via enhanced secretion of macrophage E-type PG's. Such a regulation of lysozomal enzyme secretion may also be linked to lymphokine activity as lymphokines enhance macrophage adenylate cyclase generation (Remold - O'Donnell and Remold, 1974). This picture, however, is contradicted by the reported enhancement of lysozomal enzyme release from macrophages by lymphokines (Pantalone and Page, 1975) (although the enzyme release may occur prior to the generation of sufficient PG levels) and reports that indomethacin enhanced macrophage adenylate cyclase activity (Remold - O'Donnell and Remold, 1974). The doses of indomethacin used in the latter study (3×10^{-6}M) were considerably higher than that necessary to completely inhibit macrophage PGE generation in tissue culture (Bray and Gordon, 1978) so that it remains possible that the reported action of indomethacin was not solely due to PG synthesis inhibition.

A recent paper demonstrating that PGE_1 and E_2 directly inhibited phagocytosis of zymosan by mouse, bone marrow derived macrophages and also suppressed chemiluminescence, an indicator of microbicidal activity, provides further evidence for an autoregulatory system involving macrophage derived PG's (Weidemann, Peskar, Wrogemann, Rietschel, Staudinger and Fischer, 1978).

The involvement of macrophage derived prostaglandin in chemotaxis deserves some comment. Prostaglandin E_1 has been reported to be chemotactic for rabbit neutrophils (Kaley and Weiner, 1971; McCall and Youlten, 1973) although neither PGE_1 nor E_2 were chemotactic for guinea-pig or human neutrophils or monocytes (Bray and Franco, 1978; Turner, Campbell and Lynn, 1975) or rat neutrophils (Kitchen, Boot and Dawson, 1978). Some explanation of these discrepancies has been provided by Smith and his coworkers who concluded that PGE_1 itself was not chemotactic although some metabolic product formed during storage was (Ford-Hutchinson, Smith and Walker, 1975). Potentially of greater interest have been the reports that E-type PG's were capable of inhibiting the chemotactic responses of neutrophils to stimuli such as E. coli culture filtrates (Rivkin, Rosenblatt and Becker, 1975) or casein (Bray - unpublished observations). The action

of PGE_2 in these systems appears to be via an increase in cAMP and there is also the possibility that cAMP/cGMP levels could modulate the chemotactic response as $PGF_{2\alpha}$, a stimulator of intra-cellular cGMP was capable of enhancing neutrophil chemotactic responses to bacterial culture filtrates (Hill, Estensen, Quie, Hogan and Goldberg, 1975). Such observations provide a possible mechanism for the regulation of cellular infiltration into sites of antigen activity. Ward and his co-workers have reported that E-type PG's inhibit the response of guinea-pig cells to chemotactic lymphokine (Ward et al., 1970), however, the PG doses used were high and these results were not consistent with results from this laboratory (Bray and Franco, 1978) demonstrating the inability of doses of PG's up to 1.0 ug/ml to modify neutrophil or monocyte chemotactic responses to preformed lymphokine, thus the question of PG modulation of LK chemotactic activities remains to be clarified.

Finally, the ability of macrophages to metabolise arachidonic acid raises the possibility that in response to lymphokine such cells could generate chemotactic products of lipid metabolism such as thromboxane B_2, chemotactic for mouse neutrophils and monocytes (Kitchen et al., 1978), or HETE, a hydroxy acid derivative of the lipoxygenase pathway of arachidonic acid metabolism, which has been shown to be chemotactic for human neutrophils (Turner et al., 1975).

5) PROSTAGLANDIN FEEDBACK INHIBITION IN CHRONIC DISEASE THERAPY

The ability of PGE_2 derived from inflammatory macrophages to inhibit lymphocyte generation of lymphokine in response to specific antigen raises questions as to the role of such a system in the pathology of chronic inflammatory disease.

There are a large number of disease conditions associated with cell mediated immunity (See Section 1: Introduction, Table 1) and thus possibly subject to influence by drugs which may modify lymphokine or prostaglandin activity. In 1971 Vane proposed that the anti-inflammatory actions of aspirin and other non-steroidal anti-inflammatory drugs (NSAID's) were due to inhibition of endogenous PG generation at the site of inflammation (Vane, 1971). In the context of this thesis inhibition of PG synthesis could lead to an enhancement of cellular infiltration, lymphokine generation and enzyme secretion. The possibility that such events may indeed occur during NSAID

therapy has been demonstrated by results from patients during treatment showing high levels of lymphokine in the rheumatoid joint (Stastney, et al., 1975) and enhanced levels of MIF secretion by isolated rheumatiod synovial membranes (Maini, Horsfall and Roffe, 1979). As mentioned previously there is also evidence that lymphocyte, derived factors will stimulate the generation of E-type prostaglandin by adherent cells isolated from human rheumatoid synovium (Dayer, Robinson & Krane, 1977; Dayer et al., 1979).

Thus whilst NSAID's have been shown to be active at inhibiting prostaglandin synthesis by rheumatoid tissue in vitro (Kantrowitz, Levine and Robinson, 1975) there appears to be some discrepancy between the ability of NSAID's to inhibit PG synthesis and their anti-rheumatic activity. Thus in vitro indomethacin is considerably more potent than aspirin in inhibiting macrophage PGE_2 synthesis (Bray and Gordon, 1978), however, aspirin is generally the drug of choice in therapy. Similarly the newer NSAID's such as Benoxaprofen have little PG inhibitory capacity but a high therapeutic index.

It has recently become apparent that whilst NSAID's exert some anti-inflammatory actions via PG synthesis inhibition they may also act on the lymphocyte mediated immuno/inflammatory system thus explaining other aspects of their anti-inflammatory activity seemingly questioned by the anti-inflammatory role of E-type PG's. Thus in vivo treatment with therapeutic doses of aspirin inhibits the in vitro transformation response of human lymphocytes to PHA (Crout, Hepburn and Ritts, 1975) as does treatment with high doses of aspirin in vitro (Panush and Anthony, 1976). The doses of drugs used in these studies were 2-3 orders of magnitude higher than the doses needed to inhibit PG synthesis in vitro (e.g. aspirin had an ID_{70} of 1.1 ug/ml in the macrophage model (Bray and Gordon, 1978) whereas 70% inhibition of lymphocyte transformation in vitro was achieved with a dose of 100-150 ug/ml (Panush and Anthony, 1976)). High doses of NSAID's have been shown to inhibit human leucocyte migration in vitro (Brown and Collins, 1978) and rat peritoneal cell accumulation in vivo (Di Rosa, Sorrentino and Parante, 1972). These results indicate that NSAID's whilst causing symptomatic relief of chronic disease via inhibition of PG synthesis may also have effects on lymphocyte responses unrelated to inhibition of PG generation which would overcome the possible disadvantages of PG

synthesis inhibition. A possible PG independent effect of indomethacin may be to inhibit a cAMP dependent protein kinase as the drug is active at very low doses (10^{-8}M) (Kantor and Hampton, 1978).

6) PROSTAGLANDIN FEEDBACK INHIBITION AND THE PATHOLOGY OF CHRONIC DISEASE

The inter-relationship of lymphocytes and macrophages has linked the production of lymphokines, prostaglandins and lysozomal enzymes, all mediators of acute and chronic inflammation. Such a system provides a novel model for the chronicity of inflammation and indicates a mechanism for pathogenicity that can accommodate a variety of aetiological agents. In 1974 Morley proposed that the chronicity of rheumatoid arthritis could be due to a breakdown in the macrophage/PG mediated inhibition loop. Such a breakdown would occur if the responding T-lymphocyte population was unable to react to the presence of E-type PG's and would lead to overproduction of lymphokines and thus PG's and lysosomal enzymes. There are several mechanisms whereby such defective reactivity could occur including loss of a PG receptor such as occurs with beta receptors in bronchial smooth muscle responding to isoprenaline (Benoy, El Fellah, Schnieder and Wade, 1975). Other mechanisms include a modification of the cAMP second messenger system either by induction of phosphodiesterase (the cAMP metabolising enzyme) as occurs in synoviocytes responding to PGE_1 (Newcombe, Ciozek, Ishikawa and Fahey, 1975) or loss of the anabolic enzyme adenyl cyclase which may be genetically controlled as occurs in cultured mammalian cell lines (Bourne, Coffino, Hochman, Insel, Jones, Melmon and Tomkins, 1975). The possibility that a loss of PGE reactivity occurs in rheumatoid arthritis is indicated by the high lymphokine, PG and enzyme levels in the rheumatoid joint and the relative ineffectiveness of many NSAID's to control the chronic inflammatory lesion. The existence of this type of defect has been demonstrated in other inflammatory diseases, for instance, patients suffering from active multiple sclerosis (MS) where Kirby and her colleagues have demonstrated that MIF generation by lymphocytes from MS patients was not inhibited in the presence of E-type PG's (Kirby, Morley, Ponsford and McDonald, 1976) although the response to mitogens continues to be inhibited by PG's (Goodwin and Messner, 1978). In support of a role for prostaglandins in multiple sclerosis there appears to be some evidence for the presence of an inhibitory prostaglandin modulated feedback loop present in experimental

allergic encephalomyelitis (EAE) as rats and guinea-pigs with EAE, when treated with essential fatty acid (EFA) precursors of prostaglandins show reduced symptoms of disease (Meade, Mertin, Sheena and Hunt, 1978). Splenectomy abrogated the effects on animals treated in this way (Mertin and Stackpole, 1979) and the spleen appears to be a major source of immunoregulatory prostaglandins (Webb and Osheroff, 1976). Finally, in this context, patients with systemic scleroderma show defective lymphocyte reactivity to PG's in terms of cell migration inhibition (Kelly, Miller, Rodnan and Hagmann - submitted for publication, 1979).

A breakdown in the feedback loop provides a mechanism for maintenance of chronicity of disease and whilst it would be unreasonable to ascribe all the clinical symptoms of a disease such as RA to breakdown of lymphocyte PG reactivity such a defect could contribute to the chronic pathogenesis of this disease as it allows for a range of possible aetiologies whether immunological or microbiological and provides an alternative view of chronicity unrelated to the presence of a persistant antigen or to autoimmune processes.

Breakdown of the lymphocyte/macrophage relationship provides one mechanism for chronicity, however, a second possibility could be an overreactive inhibition causing a reduction in the normal cell mediated immune response. Patients with Hodgkins disease have depressed cellular immunity both in vivo and in vitro and recently Goodwin and his co-workers demonstrated that a circulating "suppressor" cell generated large amounts of E-type PG's and that treatment with indomethacin in vitro restored the responsivness of lymphocytes from Hodgkins patients to mitogens such as PHA (Goodwin, Messner, Bankhurst, Peake, Saiki and Williams, 1977). The "suppressor" cell was glass adherent and generated about four times as much PG's as normal cells during 48 hour culture. Removal of these cells from the peripheral blood monocyte population restored the reactivity of the rest of the population to mitogens indicating that these cells were capable of modulating the T-cell responses. Such data could explain the observed impairment of lymphocyte transformation (Hersh and Oppenheim, 1968) and enhanced levels of suppressors of chemotaxis noted in patients with Hodgkins disease (Ward and Berenberg, 1974).

Finally, the description of a system linking the lymphokine generating

T-lymphocyte to the enzyme secreting macrophage via a prostaglandin mediated negative feedback loop demonstrates a new approach to the study of chronic inflammatory mechanisms and provides a simple *in vitro* model system for the evaluation of anti-inflammatory drugs whether immunosuppressive or anti-allergic.

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SECTION 6: APPENDICES

i) APPENDIX A

a) THE CAPILLARY TUBE GUINEA-PIG PERITONEAL EXUDATE CELL MIGRATION TEST: SOURCES OF VARIATION

There are a number of sources of variation relating to the cell migration test and in order to both optimise and standardise the assay several such variables have been subject to comparison and statistical analysis in order to determine their possible effect on the assay.

1 VARIATION IN (a) SOURCE, (b) SEX, (c) IMMUNE STATUS AND (d) AGE OF DONOR GUINEA-PIGS

a) Dunkin-Hartley guinea-pigs purchased from two sources (Hargreaves - Source A; Redfern - Source B) were compared and the area of control cell migration used as the response parameter. The data was subjected to a non-parametric rank test for differences between the two groups as a normal distribution was not assumed thus making the data unsuitable for parametric analysis. The Wilcoxen (or Mann-Whitney) rank test was used and provides a suitable tool for comparing groups of non-parametric data (Colquhoun, 1971).

Figure Ala illustrates the range of responses and, whilst the mean area of cell migration was greater in cultures where the cells were derived from source B, application of the two-tailed rank test indicated that the difference between the two groups was not significant ($p > 0.2$).

Source A guinea-pigs were generally used during this study as they were more readily available and less costly than Source B animals.

b) A comparison was made between the sex of the donor guinea-pig and area of control cell migration as there was the possibility that changes in the blood hormone levels of female guinea-pigs during the oestrus cycle may modify peritoneal exudate cell function or motility. Applying the Wilcoxen rank-sign test to a series of control migrations no significant differences between the sexes could be demonstrated in terms of area

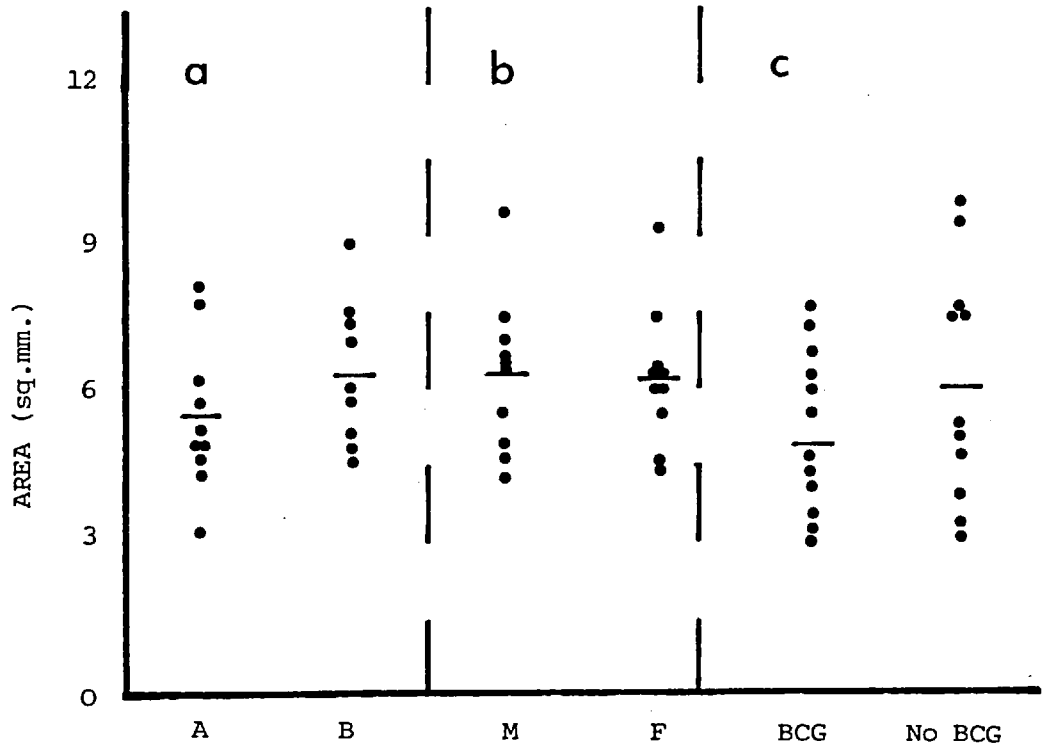

FIGURE A1 COMPARISON OF THE AREA OF CONTROL PEC MIGRATION BETWEEN (A) SOURCE A AND B GUINEA PIGS; (B) MALE AND FEMALE GUINEA PIGS; (C) IMMUNISED AND NAIVE GUINEA PIGS

(a) PEC from 3-6 month old source A and B animals were cultured for 40h. Cells were induced with starch (2-3 days). Viability>90%. Cells cultured at 3 - 4 x 10^6 PEC/tube in Eagles MEM plus 10% heat inactivated foetal calf serum (FCS). Results are expressed as area of migration in sq.mm. Each point represents the mean of 6-10 replicate cultures (No.of points, n=10). Mean areas A: 5.47, B: 6.37 sq.mm. Applying the Wilcoxen Sign Rank test for non-parametric data $p > 0.2$: Not significant (NS).

(b) PEC from either male or female source A animals were cultured as for (a). Results are expressed as in (a) and each point represents the mean of 5-10 replicate cultures. (n=10). Mean areas: Male 6.37. Female 6.32 sq.mm. Wilcoxen test: $p > 0.2$ (NS).

(c) Control cell migration of PEC from animals immunised four weeks previously to BCG were compared to PEC from unimmunised animals. Culture conditions were as for (a). Results expressed as in (a). Each point represents the mean of 6-10 replicate cultures. (n=12). Mean areas. BCG: 5.3 sq.mm. No BCG: 6.01 sq.mm. Wilcoxen test: $p > 0.2$ (NS).

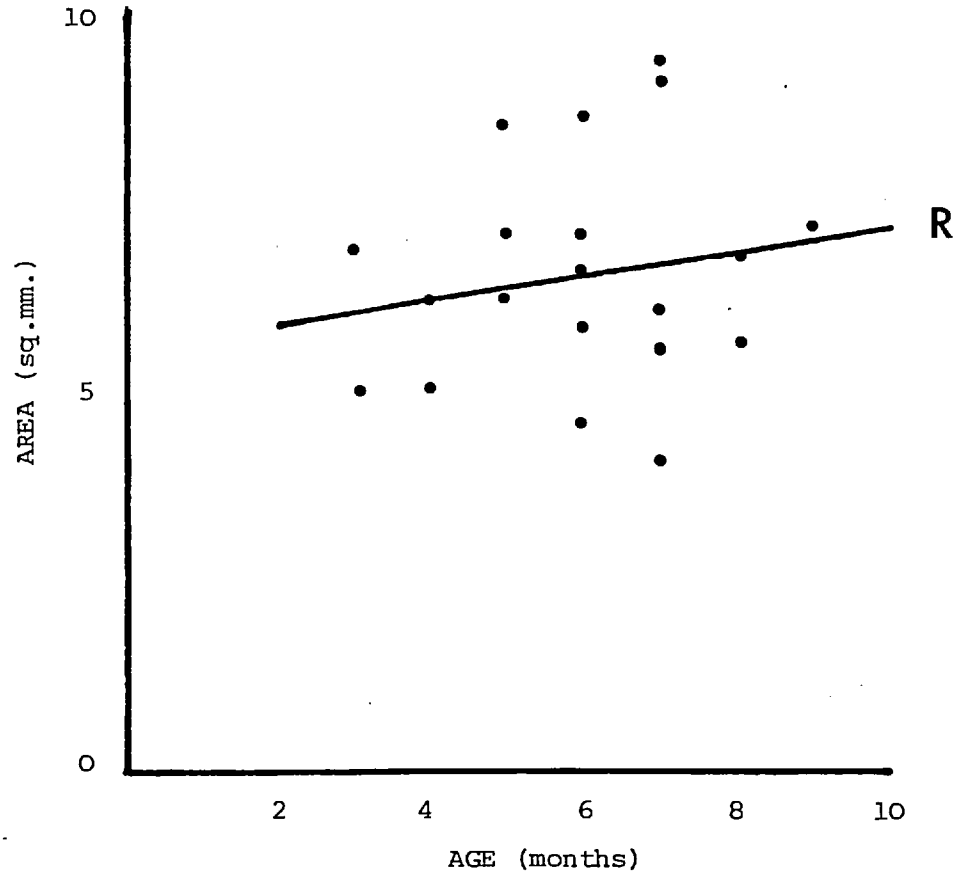

FIGURE A2 EFFECT OF THE AGE OF DONOR GUINEA-PIGS ON THE AREA OF UNTREATED PEC MIGRATION

PEC from male source A Guinea-pigs of varying ages were cultured for 40 hours in E-MEM + 10% FCS heat inactivated. PEC were induced with starch (2-3d) were >90% viable and were cultured at $3 - 4 \times 10^6$ viable cells/tube. Results are expressed as area of migration in sq.mm. Each point represents the mean of 5-10 replicate cultures. No. of points = 20.
The product-moment correlation coefficient for this data = 0.16. Students t-test: $p = 0.51$ (for 18 degrees of freedom(df): NS).
Equation of the regression line (R) : $Y = 5.76 + (0.14X)$.

of cell migration ($p > 0.2$) (Figures A1b). In general male guinea-pigs were used as for other unrelated reasons they were the most readily available donor animals.

c) Animals immunised with tuberculin (BCG) were compared to unimmunised animals in order to determine whether the migration of untreated macrophages could be modified as a result of immunisation procedures. Applying the rank-test to the results obtained no significant difference in area of control migration between immunised or non-immunised donor guinea-pigs could be detected ($p > 0.2$) (Figure A1c).

d) Source A guinea-pigs of different ages were used to provide cells for the migration assay and the age of the animal was compared to the area of control cell migration. The groups of observations were examined to detect any correlation between age and cell migration area by determining the product-moment correlation coefficient (pm.cor.) and subjecting the result to a two-tailed Students t test of significance. There was no apparent correlation between age and cell migration (pm.cor. 0.16; Students t test $p > 0.5$) within the age limits of donor guinea-pigs used as cell sources (3-9 months) (Figure A2). As a result of the data shown here the age of donor animals was not taken into account in routine cell migration assays.

2 VARIATIONS IN (a) PERITONEAL EXUDATE CELL INDUCTION, (b) CELL VIABILITY AND (c) CELL NUMBERS

a) A variety of irritant materials have been used in different laboratories to induce peritoneal inflammatory cell accumulation and both the type of material used and the length of time the irritant is present in the peritoneal cavity may modify the numbers and proportions of cell types present in the fluid exudates. In order to ascertain the relative numbers of different cell types present following different inflammatory stimuli, sample cell exudates were subjected to cytocentrifugation (Cytospin: Shandon, UK), fixed in methanol and stained with Giemsa stain. In order to categorise the cell types present the following morphological and staining criteria were adopted:

1 Macrophage like: Large cells (10-30μ) with an indistinct cytoplasmic

membrane, highly vascular basophilic cytoplasm and a lightly basophilic kidney shaped nucleus (25-30% of the total cell volume).

2 Lymphocyte like: Small cells (8-13μ) with a distinct cytoplasmic membrane, little visible cytoplasm and a dark staining basophilic nucleus occupying 60-90% of the intracellular space.

3 Neutrophil like: Small cells (10-20μ) with a distinct cytoplasmic membrane and a blue staining multi-lobed nucleus occupying 50-60% of the intracellular space.

4 Other cell types occasionally seen included large polymorphonuclear leucocytes with an eosinophilic cytoplasm and large peritoneal wall lining cells with abundant basophilic cytoplasm and a small round nucleus.

All the peritoneal exudates examined contained red cells with numbers varying from 5-20% of the total cell populations. Occasionally red cell contamination was severe (up to 90% of all cells) and such exudates were discarded.

All the inflammatory stimuli examined induced predominantly macrophage rich exudates with variable numbers of neutrophils and 10-20% lymphocytes (Table A1). From the data presented for the variations in day of sacrifice it can be seen that, for starch and oil, the ratio of macrophages to neutrophils increases with time. Areas of cell migration are largest using two day exudates, however the differences between two, three and four day exudate migrations was not significant ($p > 0.20$ Figure A3). For the majority of routine studies animals were injected with starch and the cells harvested after three days. This regimen proved the most convenient and the use of starch rather than oil meant that the cell exudates required less washing prior to use.

Cell numbers did not vary greatly between the different treatments (Table A1).

b) The viability of guinea-pig peritoneal exudate cells was assessed routinely by vital-dye exclusion (See Section 2: Methods, vi) (a)) prior to culture. A series of 23 samples from starch induced peritoneal exudates

TABLE A1 CELL TYPE AND NUMBER DERIVED FROM THE GUINEA-PIG PERITONEAL CAVITY FOLLOWING INJECTION OF INFLAMMATORY STIMULI

ip. Stimulus (in 20ml inj. volume)	Days prior to sacrifice	n	M % ± SD	N % ± SD	L % ± SD	Others % ± SD	Mean No. of viable cells ($X10^8$)/animal*
2% starch solution	2	10	50 ± 5.1	40.2 ± 4.6	8.2 ± 0.8	1.6 ± 0.2	1.7
	3	10	65.4 ± 7.3	20.1 ± 3.7	13.0 ± 3.1	1.5 ± 0.5	1.8
	4	10	79 ± 4.7	6.6 ± 1.1	12.2 ± 2.7	2.2 ± 0.4	0.9
Light mineral oil	3	10	68.1 ± 8.3	10.6 ± 1.2	19.7 ± 4.6	1.6 ± 0.1	1.6
	4	10	81.3 ± 10.1	6.7 ± 0.9	11.1 ± 3.7	0.9 ± 0.2	1.9
Proteose/peptone 10% W/V	4	4	60.1 ± 12.1	30.4 ± 7.4	6.5 ± 2.0	3.0 ± 0.6	1.3
Thioglycolate Broth (40.5 g/l)	4	4	53.2 ± 8.1	40.1 ± 6.5	6.0 ± 0.9	0.7 ± 0.2	0.96

SD: Standard deviation. n = number of animals

* Cell numbers were calculated using a neaubaur Heamocytometer (Section 2: Methods 2 iv) a)
M = Macrophage like
L = Lymphocyte like
N = Neutrophils
Others = Endothelial cells, Eosinophils

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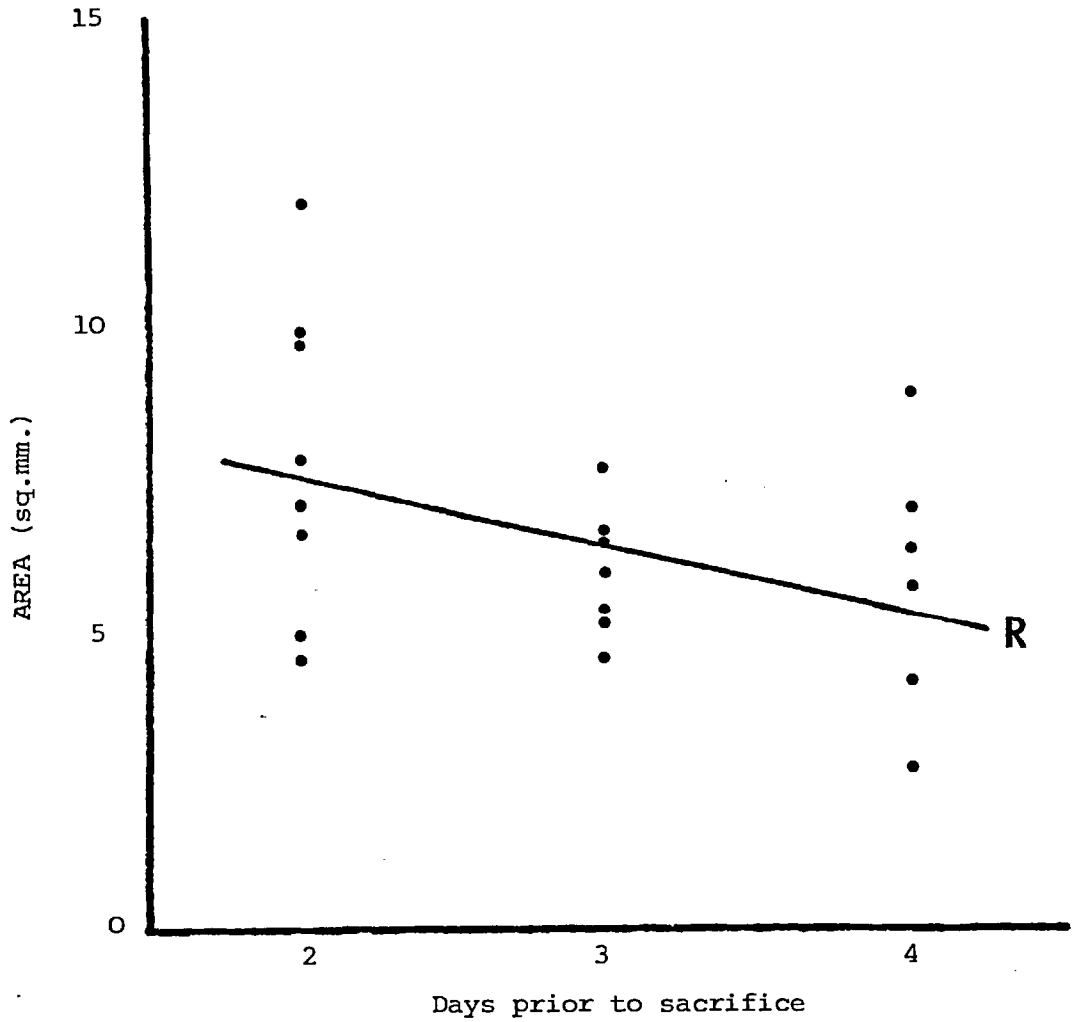


FIGURE A3 EFFECT OF THE NUMBER OF DAYS PRIOR TO SACRIFICE FOLLOWING ip. STARCH INJECTION ON THE AREA OF UNTREATED GUINEA-PIG PEC MIGRATION

PEC were taken from 3-6 month old animals injected 2-4 days previously with 20 mls 2% starch ip. Animal types and culture conditions were as in Figure A2. Results are expressed as area of migration in sq.mm. Each point represents the mean of 6-12 replicate cultures. No. of points = 23.
The product-moment correlation coefficient for this data = -0.374 (t-test: $p > 0.2$ (for 21 df): NS).
R : $Y = 8.96 - (0.95X)$.

were compared in terms of the control migration area in order to examine whether the percentage viability of PEC correlated with migration area. In this sample the mean % viability was 91% and the range 65-100%. The product moment correlation coefficient of +0.164 was not significant (Student t test: $p > 0.5$) however, there was an indication from the slope of the regression line that, as might be expected, a higher % viability was associated with larger migration area (Figure A4a). Routinely samples of PEC with a viability of 60% or less were not used in migration experiments.

c) In a study of variations in techniques used in the cell migration assay Hughes noted that increasing the length of the packed cell pellet in the capillary tubes increased the area of control emigrations (Hughes, 1972). In view of this observation a series of cell migration tests (24) with different numbers of cells in the capillary tubes were examined and the results were compared in terms of the area of control emigration. The mean cell number was 2.86×10^6 viable cells/capillary tube (range: 1.36 - 4.86×10^6 viable cells/capillary tubes). A product moment correlation coefficient of +0.537 was obtained when cell numbers were compared to area of control cell migration (Figure A4b). This positive correlation was statistically significant (Students t test: $p < 0.05$) in agreement with the results published by Hughes. During routine cell migration tests a cell concentration of $3-4 \times 10^6$ viable cells/tube was used allowing for up to 60 capillaries to be prepared from a single donor animal (Mean total cell number for a three day starch induced exudate was 1.8×10^8 viable cells: Table A1).

3 VARIATIONS IN (a) CONCENTRATION, (b) SOURCE AND (c) "INACTIVATION" OF SERA USED IN THE CULTURE CHAMBERS

a) Variations in the concentration of serum in the culture medium has been reported to greatly modify the area of cell emigration (Hughes, 1972), therefore a range of serum concentrations from 0-40% was tested. Heat inactivated (heated at 56°C for 30 mins) foetal calf serum was used and the area of control cell migration compared to the concentration of serum in the culture fluid. The results showed a highly significant positive corellation between increasing serum concentration and increased area of cell emigration within the range of concentrations tested (pm. cor: +0.829;

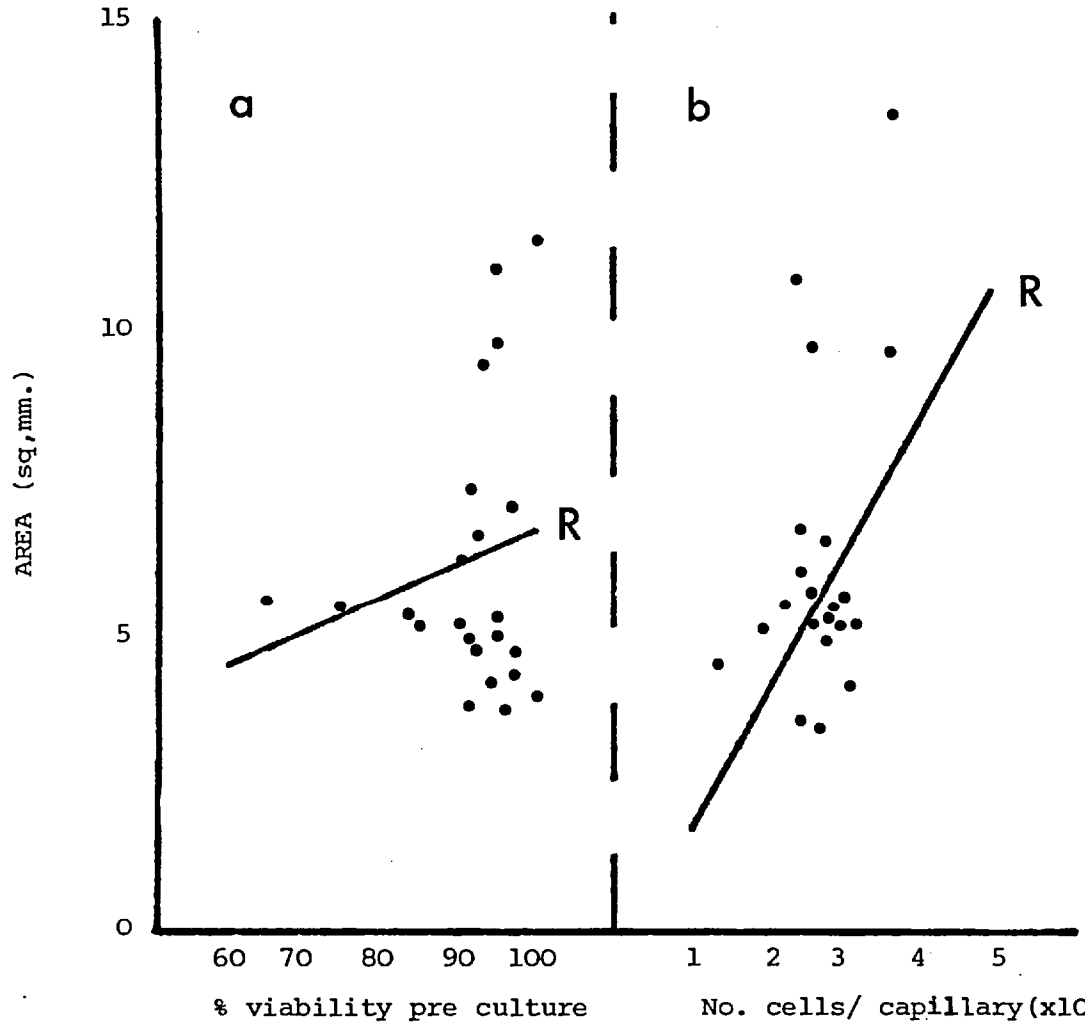


FIGURE A4 EFFECTS OF (A) PEC VIABILITY AND (B) NUMBER OF CELLS PER CAPILLARY TUBE ON THE AREA OF MIGRATION OF UNTREATED GUINEA-PIG PEC

(a) Apart from the percentage viability PEC from 3-6 month old donor guinea-pigs were prepared and cultured as in Figure A2. The cell viability was predetermined and % viability compared to area of emigration. Results are expressed as area of migration in sq.mm. Each point represents the mean of 6-10 replicate cultures. No. of points = 23.
Product-moment correlation coefficient = 0.164.
Students t-test: $p > 0.5$ (NS).

(b) Apart from the number of cells/capillary tubes PEC from 3-6 month old donor guinea-pigs were prepared and cultured as in Figure A2. Cell numbers/tube varied from 1 - 5 x 10^6 cells and cell numbers were compared to area of untreated cell migration (sq.mm.). Each point represents the mean of 5-10 replicate cultures. No. of pairs = 24.
Product-moment correlation coefficient = 0.537
Students t-test: $p < 0.05$.
R : $Y = -0.55 + (2.49X)$.

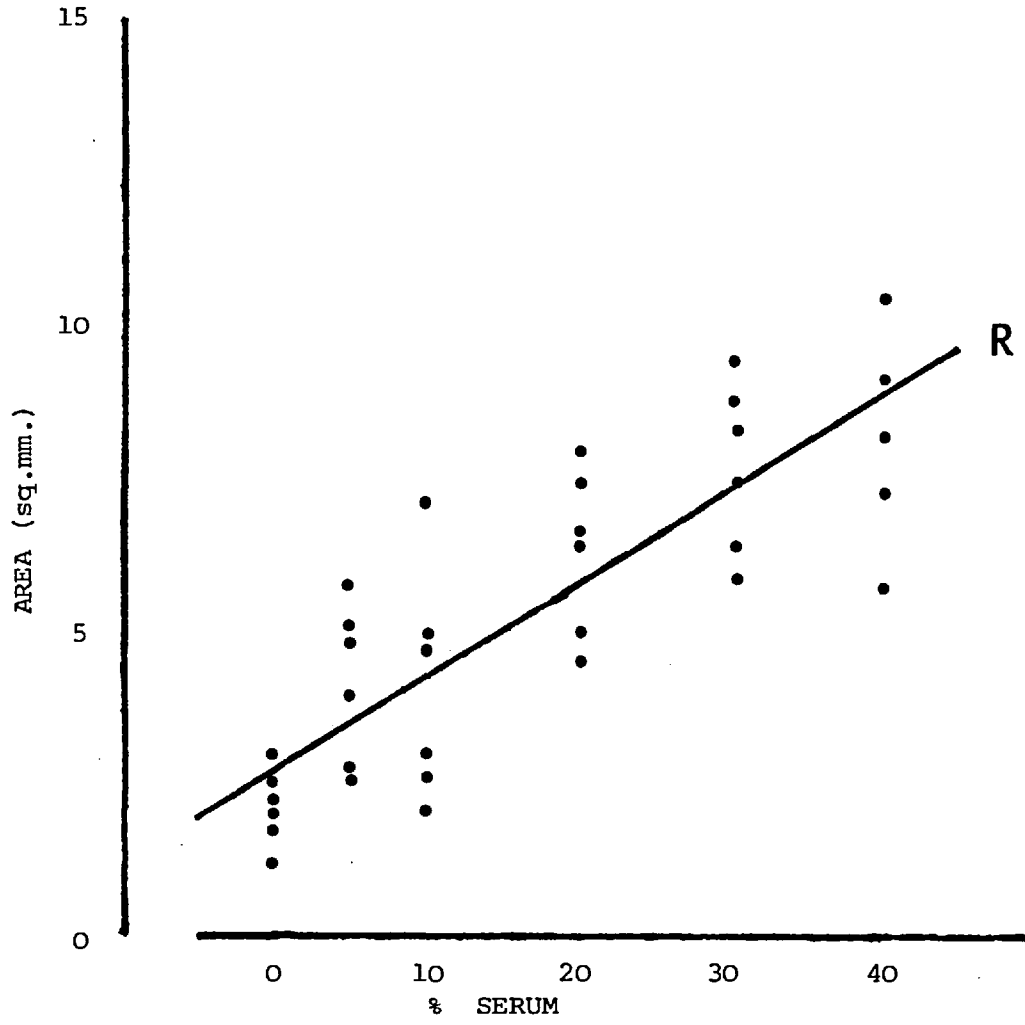


FIGURE A5 EFFECT OF VARIATION IN FOETAL CALF SERUM CONCENTRATION ON THE MIGRATION OF UNTREATED GUINEA-PIG PEC

Apart from the serum concentration PEC from 3-6 month old donor guinea-pigs were prepared and cultured as in Figure A2. Concentration of heat inactivated foetal calf serum varied from 0-40% and serum concentration was compared to area of migration (sq.mm.). Each point represents the mean of 6-12 replicate cultures. No. of points = 36.
Product-moment correlation coefficient = 0.829.
Students t-test: $p < 0.00005$.
R : $Y = 2.66 + (0.15X)$.

Student t test: $p < 0.00005$: Figure A5). During routine migration tests a concentration of 20% serum was used for reasons of economy.

b) Sera from several different sources were tested in the cell migration assay in order to evaluate the effects of sera from different species and batch variation within the same species. Homologous pooled guinea-pig sera obtained by cardiac puncture from stock Dunkin-Hartley donors was compared to commercially obtained foetal bovine serum. Sera were all heat inactivated (56°C; 30 mins) and tested at a concentration of 20% in culture medium. The effects on the area of control cell emigration are shown in Table A2. The results indicated that whilst migrations from cultures containing guinea-pig sera were consistently larger there was also a greater degree of variation between batches when compared to cultures containing foetal bovine serum (% coefficient of variation 44% for guinea-pig serum compared to 8.2% for foetal calf serum). As a result of the data presented in Table A2 routine cell migration tests were carried out using Flow foetal calf serum in an effort to standardise the assay. Several batches of sera were tested prior to routine use to ensure that the batch allowing the largest area of control cell emigration was used subsequently. During the period of this study only three separate batches of Flow sera were used in the majority of assays.

c) The possible involvement of complement components on cell migrations in the presence of foetal calf serum was tested for by comparing control cell migrations after incubation with 20% heat inactivated serum (56°C for 30 mins) with migrations in 20% non-heated serum. The results were plotted and compared using the Wilcoxen sign rank test (Figure A6). From the data presented it can be seen that migrations with heat inactivated serum in the culture medium were significantly larger ($p < 0.02$) than those cultures containing untreated serum. These results indicate that there may be a heat labile inhibitory material in commercial foetal calf serum, possibly related to complement. As a result of this comparison cell migration assays were routinely performed using sera that had been heated at 56°C for 30 mins prior to use.

TABLE A2 EFFECT OF VARIATION IN BATCHES OF SERUM ON GUINEA-PIG CONTROL CELL MIGRATION AREA

Serum batch	n	Mean area ± SD (sq.mm.)	
No serum	10	2.7 ± 0.3	
Flow Foetal calf serum			
Batch 1	10	7.1 ± 0.6	
Batch 2	6	6.7 ± 0.65	
Batch 3	8	6.9 ± 0.91	
Batch 4	8	7.4 ± 0.8	
Gibco Foetal calf serum			
Batch 1	6	6.0 ± 0.47	
Batch 2	6	6.3 ± 0.6	
Overall mean ± SD	44	6.73 ± 0.49	% Variance = 8.2
Normal guinea-pig serum			
Batch 1	6	9.2 ± 1.26	
Batch 2	8	11.3 ± 1.5	
Batch 3	8	7.1 ± 1.1	
Batch 4	8	8.6 ± 1.8	
Overall mean ± SD	30	9.3 ± 1.76	% Variance = 44

SD: Standard Deviation
n: number of replicate migration chambers
All sera were heated for 30 mins at 56°C. Culture time 40 hours

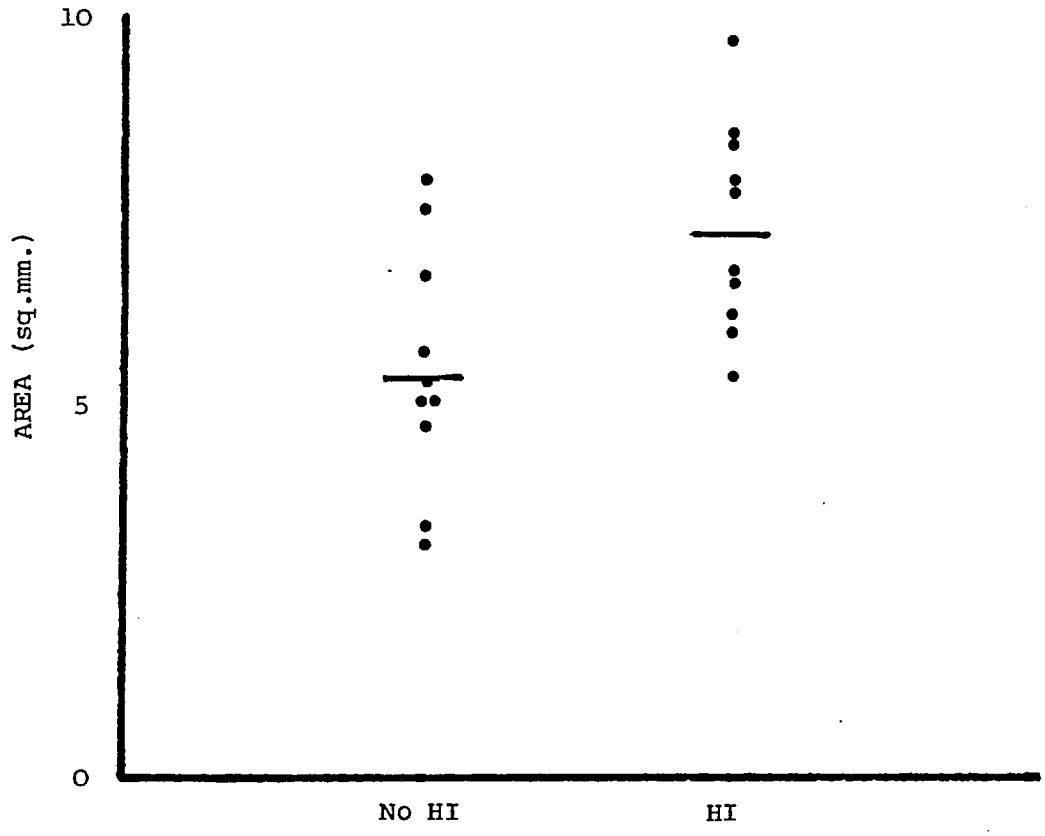


FIGURE A6 EFFECT OF HEAT INACTIVATION OF SERUM ON THE MIGRATION OF UNTREATED GUINEA-PIG PEC

Apart from the serum being either heated at 56°C for 30 minutes or untreated, PEC from 3-6 month old donor guinea-pigs were prepared and cultured as in Figure A2. Areas of migration (sq.mm.) using either heated (HI) or unheated (NoHI) serum were compared. Each point represents the mean of 6-10 replicate cultures. No. of points = 10. Mean area of migration. NoHI = 5.35 sq.mm.; HI = 7.23 sq.mm. Wilcoxen test: $p < 0.02$.

b) TESTS FOR NORMALCY AND SCEDASITICITY : JUSTIFICATION FOR USE OF PARAMETRIC STATISTICAL ANALYSIS

1 In order for two groups of data to be examined using parametric statistical analysis such as the Students t test for significance the data must be assumed to be normally distributed. Tests for normal distribution have therefore been applied to groups of cell migration data in the presence (test) and absence (control) on an inhibitor. The inhibitory agent used was the specific antigen PPD (2.0 ug/ml: ID_{50}) cultured with PEC from donor guinea-pigs sensitised to tuberculin. Data was derived from several experiments where the variables examined in the previous section were kept constant. Both control and test data were plotted (Figures A7 and A8) and the degree of skewness and kurtosis calculated as an indication of normalcy. For both control and test data neither of the above were found to differ significantly from normal (control: skewness, $p = 0.429$; kurtosis, $p = 0.301$. Test: skewness, $p = 0.248$; kurtosis, $p = 0.278$). Whilst the number of samples in each data group was small (control $n = 20$; test $n = 23$) increasing the number of samples would be expected to reinforce the results shown. From the data presented it was apparent that for control emigration and at least antigen induced inhibition of cell migration the groups of data were normally distributed thus allowing use of parametric data analysis. Based on these results Students t tests have been used where relevant to compare sets of data throughout the present study.

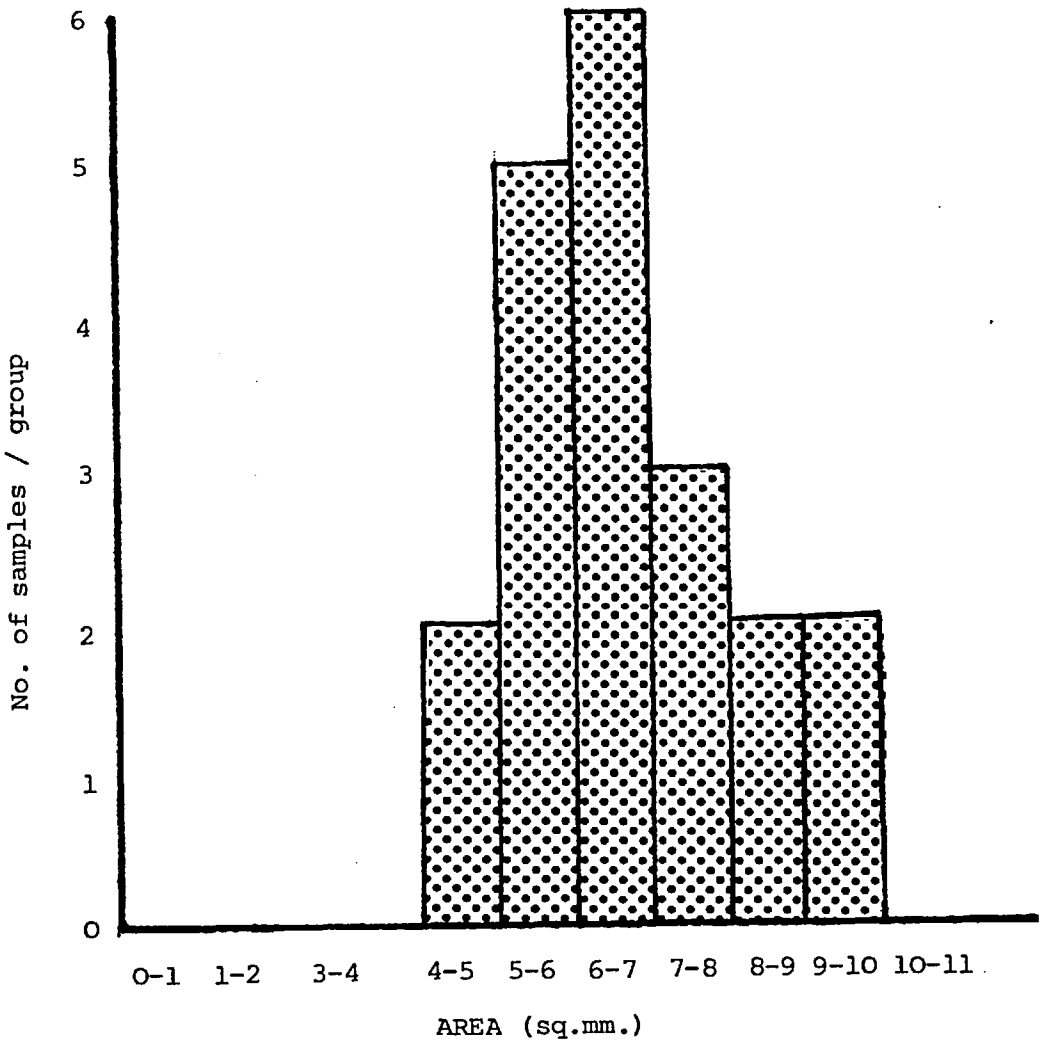


FIGURE A7 HISTOGRAM SHOWING THE DISTRIBUTION OF THE AREAS OF A SERIES OF UNTREATED GUINEA-PIG PEC MIGRATION

PEC from 3-6 month old donor guinea-pigs were prepared and cultured as in Figure A2. Each sample mean represents 6-10 replicate cultures. No. of samples = 20. Results are plotted as sample number versus control migration area (sq.mm.).
Z for Skewness = 0.18 ($p = 0.429$: NS.).
Z for Kurtosis = 0.52 ($p = 0.301$: NS.).
Mean area (95% confidence limits).
= 6.58 (2.8, 8.45) sq.mm.

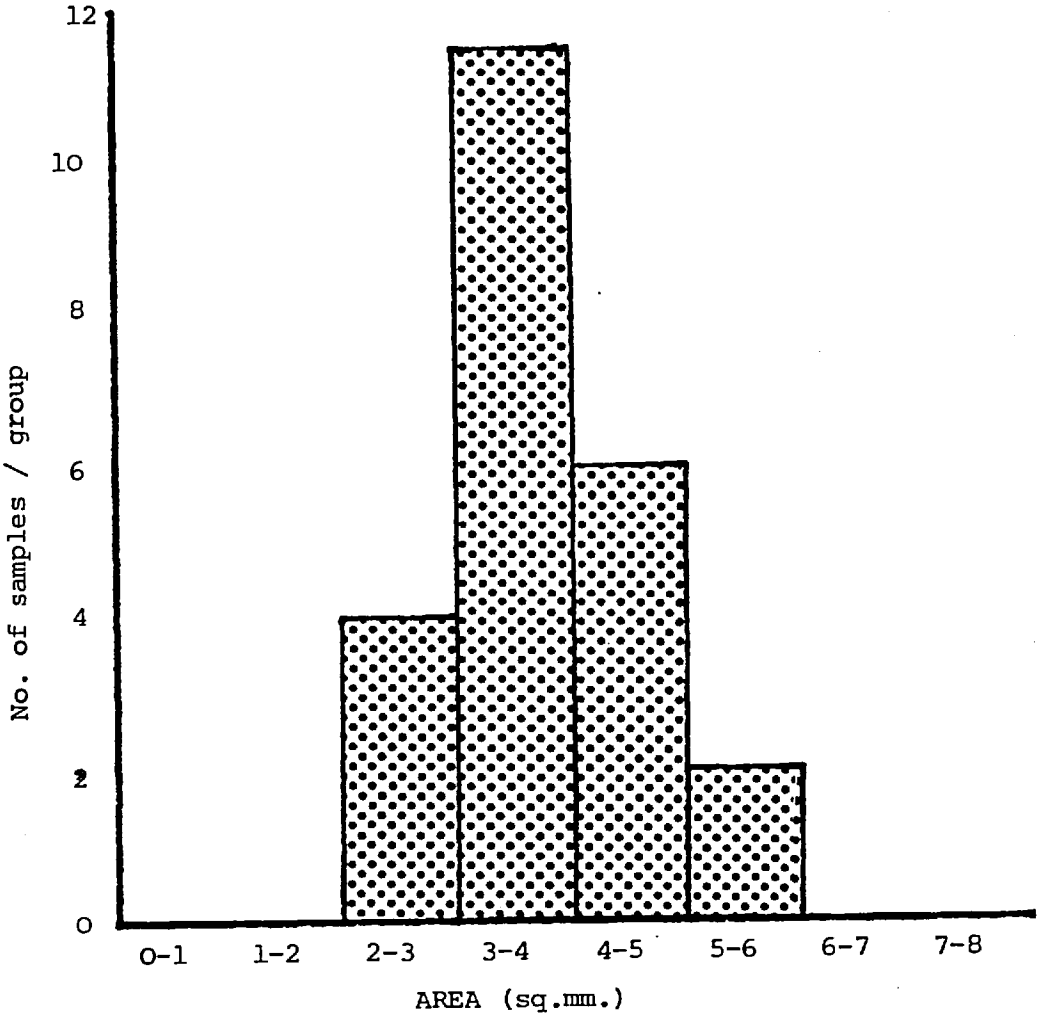
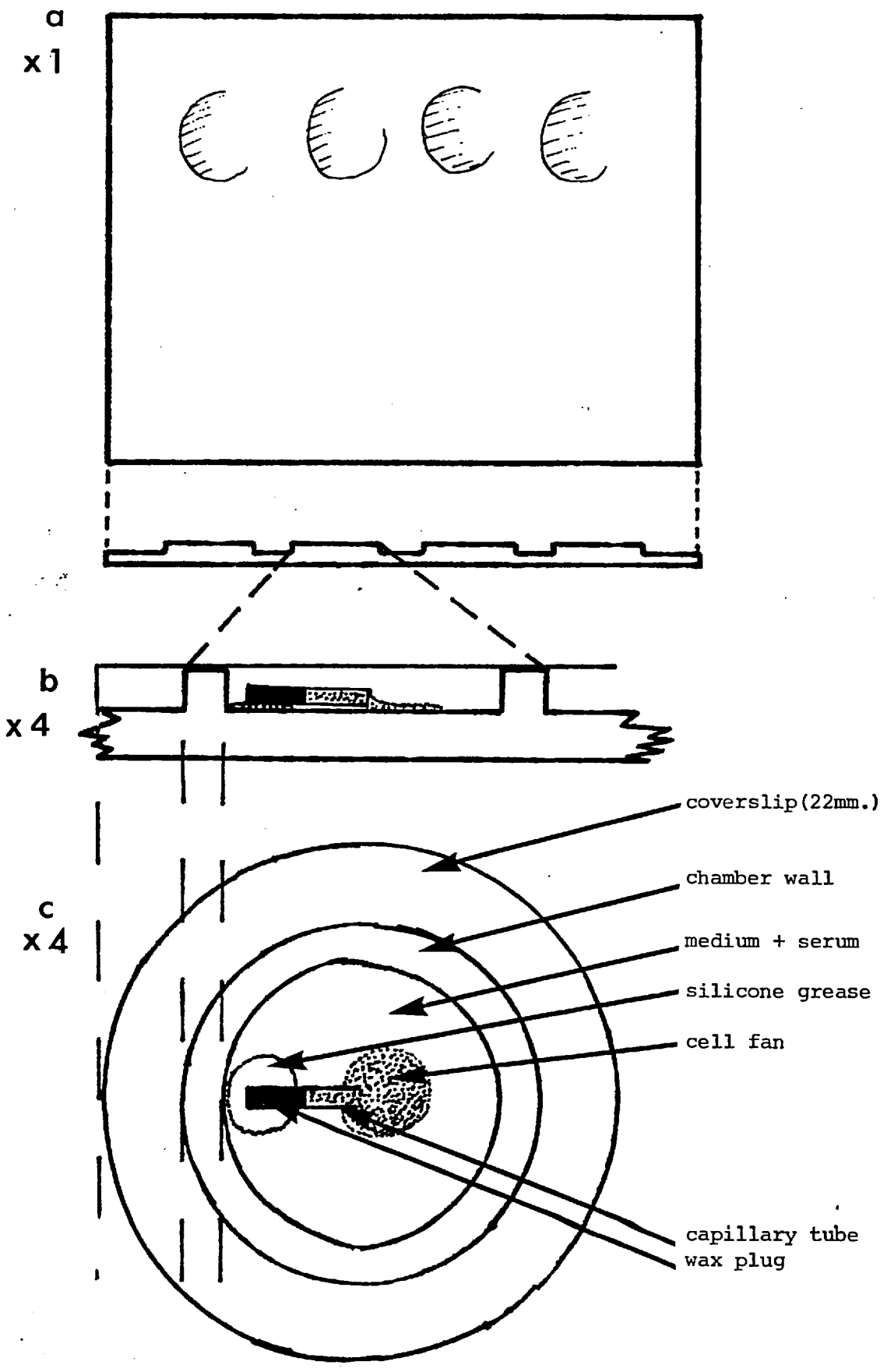


FIGURE A8 HISTOGRAM SHOWING THE DISTRIBUTION OF THE AREAS OF A SERIES OF GUINEA-PIG PEC MIGRATIONS FROM IMMUNISED ANIMALS IN THE PRESENCE OF A FIXED DOSE OF ANTIGEN

PEC from 3-6 month old donor guinea-pigs previously immunised to BCG were prepared and cultured as in Figure A2 and in the presence of 2.0 ug/ml PPD. Each sample mean represents 6-8 replicate cultures. No. of samples = 23. Results are plotted as sample number versus migration area (sq.mm.).
Z for Skewness = 0.68 ($p = 0.248$: NS.).
Z for Kurtosis = 0.596 ($p = 0.278$: NS.).

FIGURE A9 (a) Plan and elevation of Sterelin "Kennedy" cell migration plate. Enlarged plan (b) and elevation (c) of a single chamber following culture.



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Page	Line	
4-6		P.5 missing
12	4	"Kock" - Koch
13	19	"atopically" - topically
22	13	"periphal" - peripheral
35	14	"lgG" - IgG
	27	"TYPE lV" - TYPE IV
40	32	"... responses," - responses.
41	35	"Lichtenstein" - Leichtenstein
44	10	"... tuberculosum .." - tuberculosis
48	11	"PERIPHAL" - PERIPHERAL
75	14	"... antigen represent .." - LK represent
76	11 & 12	Key to be inverted, i.e.
79	15	"periphal" - peripheral
81	3	"TO TUBERCULIN" - TO MYCOBACTERIUM TUBERCULOSIS
90	12	"0.28-1.10)" - (0.28-1.10)
91	27	" ...(p=0.05)" - p<0.05
120	17	"... relative potency shifts .." - relative dose ratios
	18	"....3+3 potency ratio .." - 3+3 dose ratio
	21	"....and potency ratio .." - and dose ratio
123	11-12	"Potency ratio ... Table 8" - Dose ratio .. Table 9
124	11-12	"Potency ratio ... Table 8" - Dose ratio ... Table 9
125	2	"Potency ratio" - Dose ratio
138	9	"... ug/kg" - mg/kg
147	12	"... were 95% viable" - were >95% viable
161	9	"... cultures filtrates .." - culture filtrates
169	17	"... 10^{-4} histamine .." - 10^{-4}M histamine
	36	"... histamine of .." - histamine in
	37	"... & Grout .." - & Grant
170	17	"... by cortisone .." - by corticosteroids
187	22	ARONSON ref. to come after Andreis et al
188-189		Pages numbered and bound in reverse order
189	23	"... in cell Mediated .." - in Cell Mediated
	31	"... M.J.,MELMON .." - M.J. and MELMON
	33	BOURNE ref. to come after BOURNE et al (1974)
192	3	"GABRIELSEN .." - GABRIELSON
194	29	"KOCK" - KOCH
196	15	"... Willoughgy,P.A.).. " - Willoughby,D.A.)
198	20	"... and Le BUGLIO .." - Lo BUGLIO