FIBRIN, FIBRINOLYSIS AND THE IMMUNE SYSTEM

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THIS WORK IS DEDICATED TO

MY WIFE AND FAMILY

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Three people suggested that I should look at the problem of clotting and immunity; and I am grateful to them: Dr. Richard Bolton and Dr. Ken Gough of Bath and Dr. Peter Moorhead of Sheffield.

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CHAPTER 1

HISTORY

The fibrin equilibrium: fibrin production, fibrin and fibrinolysis has recently been considered to be involved in several clinically important immunological reactions. In organ transplant rejection and glomerulo nephritis the alteration in the equilibrium is thought to be secondary to the immunological insult. This thesis is primarily concerned with the association between neoplasia, a disturbed fibrin equilibrium and resulting impaired immunity. The association has been recognised as clinically relevant for a long time: an excellent account was given by Spath (1866) of gastric carcinoma and venous thrombosis. The incidence of neoplasia is high, and for several tumours the mortality has not significantly changed despite improved surgical and anaesthetic techniques; any simple beneficial change to the immune system may be clinically highly relevant.

The history of the association between fibrin and neoplasia can be divided into two parts; the description of changes resulting from cancerous tissues, and data suggesting an aetiologically important association between the fibrin equilibrium and neoplasia.

The abnormal tendency to develop venous thromboses in the tumour laden patient was recognised by a variety of clinicians: a perceptive account being by Schlagenhaufer (1909). Some of these patients fulfill the criteria of thrombophlebitis migrans, which can occur in the absence of neoplasia. Whether associated with tumour or not, patients with thrombophlebitis migrans have reduced spontaneous fibrinolysis which can be improved with the administration of phenformin and ethyloestrol; in association with such improvement their clinical

condition improves. Fibrinolysis is also abnormal in patients with tumours, this has best been shown in the perioperative period when patients with carcinoma tend to have a reduced spontaneous fibrinolysis and poor response to the insult of the surgery: this correlates loosely but not clinically usefully, with the incidence of deep venous thromboses. This depression of fibrinolysis has not yet been adequately explained but the increased incidence of thrombosis around the tumour is probably related to the high level of thromboplastic activity intrinsic in some cancers (Lawrence et al., 1952).

The proposition that a changed fibrin equilibrium can affect tumour growth rests on several important and early observations: Ashworth in 1869 demonstrated cells which appeared similar to those in the primary tumour in blood obtained at postmortem examination. By studying metastases and primary tumour Goldman was the first to stress the important of venous invasion by malignant cells and Schmidt in a series of perceptive observations described the frequency of tumour emboli and noted that many were surrounded by thrombus. Of the emboli several appeared quiescent, several were in mitoses, but some were in regression. He proposed that there had been intravascular destruction by a thrombotic process but with the assistance of leucocyte activity. Others developed this idea (Levin & Sittenfield 1911) to include blood plasma inhibitory substances. Tedenuma (1923) in experiments on fowl sarcoma, found that while this tumour rarely produced metastases under normal conditions, metastases appeared frequently if the tumour burdened fowl was repeatedly bled; he attributed this to the loss of some defensive agent in the blood.

Unfortunately the experimental workers who advocated this view ignored other less hypothetical factors. The older methods of preparation of tumour emulsions entailed lethal trauma to much of the tumour and Weil showed that several early results (such as Levin & Sittenfield obtained) were likely to have resulted from the technique of preparation. In the experiments of Tadenuma the repeated bleeding of animals was a drastic procedure provoking repercussions in other tissues some of which could have been responsible for augmented metastasis. Certainly these considerations prompt caution before attributing intravascular degeneration to any specific anti-cancerous quality residing in blood clot. Further development of ideas about the fate of intravascular metastases awaited the development of the rabbit ear chamber and high magnification, high resolution, cinemicrographic in vivo analysis of cancer cell endothelial surface interaction (Wood 1958). There is a prompt, firm direct attachment of tumour cells to vascular endothelium of postcapillary venules. A blood clot is formed around the cancer cells: these move through endothelial defects into the perivascular connective tissue where they proliferate. Loosely attached tumour cells apparently die. Increased fibrin production or platelet stickiness enhances metastasis formation whereas factors producing platelet dysfunction, thrombocytopenia, or fibrinolysis decrease metastases.

Heparin and Warfarin, the two classical anticoagulants, have been shown to reduce metastasis formation. Heparin is produced by mast cells and probably by other cells of the reticuloendothelial system and is a sulphated poly-saccharide. It has been shown to have direct effects upon normal and neoplastic cells by inhibiting mitosis (Kudrjashovetal 1969) probably by preventing the cytoplasm gelation

state which occurs prior to metaphase (Heilbrunn & Wilson 1949). The anionic character of Heparin may be of importance in preventing attachment of tumour cells to the endothelium by electrostatic forces. Unfortunately, this same hypothesis could be extended to suggest that adhesive forces between cells is decreased producing an enhanced release of tumour emboli into the circulation (Hagmar & Boeryd 1968). Warfarin uncouples phosphorylation which interferes with cellular metabolism and hence mitosis, and indeed may be cytotoxic (Lisnell & Mellgren 1963). Certainly movement of tumour cells is reduced by exhibition of Warfarin (Thornes et al., 1968). With small doses of IV tumour in the experimental animal both Heparin and Warfarin reduce the incidence of metastasis in the first capillary bed (Thornes et al., 1968). This effect was lost in larger doses of tumour. Spontaneous metastasis from a growing tumour were not influenced by Heparin therapy although some experiments suggested an increased number of metastases. Warfarin therapy decreased metastasis formation and reduced the growth rate of some primary tumours. Some of these observations may be due to an enhanced immune response; this was suggested by the experiment of Kiricute et al (1973): the incidence of metastases in four experimental animal groups were 83% in controls, reticulo endothelial (RE) nonspecific stimulation 76%, Heparin 47% and RE stimulation with Heparin 28%. Even with these results the suggested mode of action of Heparin was by preventing adhesion of tumour cells to the vascular endothelium.

Undoubtedly Heparin, and to a lesser extent, Warfarin has a known influence on the immune system. Heparin has an anti-complement activity, probably due to anionic inhibition of the esterases in this enzyme cascade; it possesses an anti-inflammatory activity on local or

systemic administration, supposedly benefiting asthma, lupus erythematosus, allergic thrombo-cytopaenia, haemolytic anaemia, dermatitis herpetiformis, exzema, rheumatoid arthritis, allergic glomerular nephritis and iritis. Several of these diseases are thought to have an immunological aetiology. Warfarin therapy increases polymorphonuclear leucocyte, and later macrophage, congregation around the neoplasm, though lymphocyte activity is suppressed. Similarly skin reactions for delayed hypersensitivity are reduced and show reduced numbers of infiltrating cells.

Thornes and co-workers used a protease derived from Aspergillus oryzae in the treatment of cancer and leukaemia; this had a direct proteolytic action but also reduced the concentration of plasmin and trypsin inhibitors present in the plasma. In patients with leukaemia, if antiplasmin levels were reduced to less than 50% of normal, leukaemic cells were killed in vivo. This did not occur if Brinase were added in vitro. Six cases given daily Brinase for 10 to 30 days developed complement-dependent autocytotoxic antibodies to lymphocytes and leukaemic cells. Repeated therapy required the same number of days to reproduce the autocytotoxicity. By chance, a patient with carcinoma of the lung was given Brinase on the same day as the skin tests were repeated to test for delayed hypersensitivity. He was previously anergic, but developed strongly positive reactions to PPD, streptokinase/dornase, and phytohaemagluttinnin (PHA). This finding was replicated in a further four patients, previously anergic. Early work was performed on cellular immune reactions and fibrinolysis. T lymphocyte numbers were assessed in various patients and found to be correlated with antiplasmin levels. Brinase treatment of anergic individuals increased the T lymphocyte response by 50-150%.

Streptokinase in twenty patients similarly increased T cell count (in 19 out of 20). Brinase has also been found to affect leucocyte motility; following Brinase infusion migration of leucocytes increased by about 80%. Clinically these effects have been assessed in the Bowel Tumour Study; patients undergoing resection for carcinoma of the large bowel were divided into either of two groups, placebo or active. The latter were given perioperative streptokinase, sufficient to enhance fibrinolysis; so far the results are suggestive of prolonged survival (5 year survival 61% in streptokinase treated, 29% in placebo). The site of action of this effect has been postulated to be either complement activation, macrophage lysosomal activation or by removing blocking substances from about either target cell or macrophage-lymphocyte.

Fibrin preventing drugs, and fibrinolytic agents have both been of clinical use, although if more were known of their mode of action drug administration and timing may be more logical, and effective.

There are, moreover, other reasons to assume a connection between fibrin equilibrium and the immune system: coagulation and fibrinolysis share common serine protease enzymes and zymogens with the complement cascade, and with the Kall kirein system (Figure 1.). It is thought that Hageman factor XII initiates not only the intrinsic clotting reaction to foreign surfaces but also Kinin-forming and fibrinolytic response; and hence the complement system (Donaldson 1968). This factor was demonstrated by its absence (Ratnoff & Colopy 1955) in a patient with a greatly prolonged clotting time in vitro. To induce clotting, activation of XII allows an

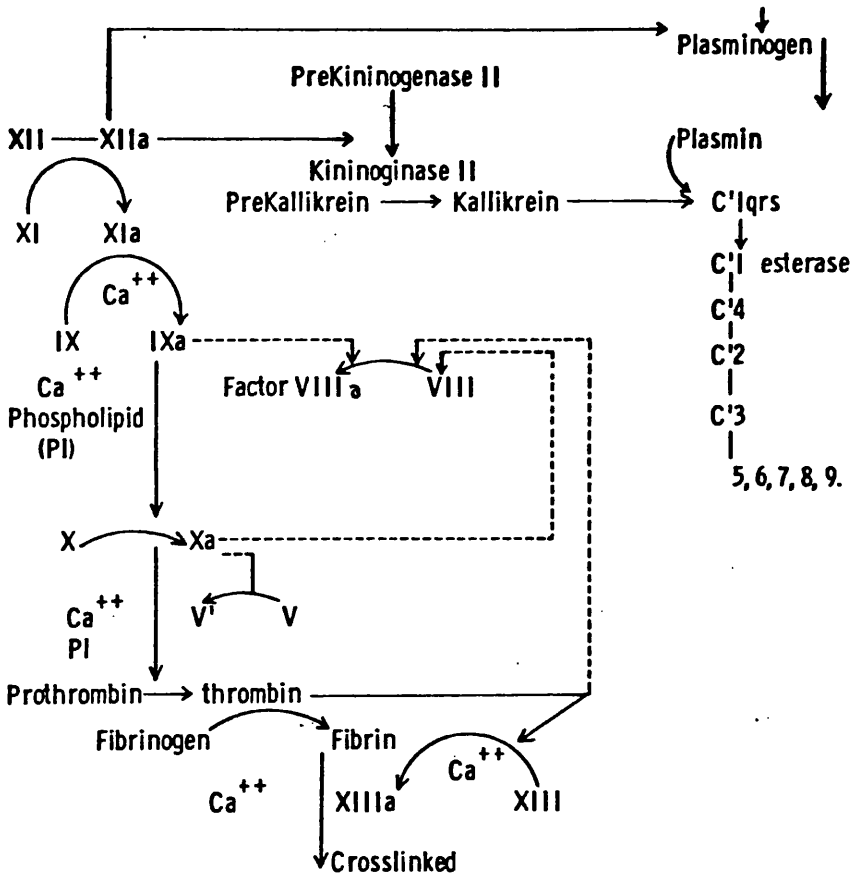


Fig. 1 : Coagulation, Kinin, Plasmin, Complement cascades.

amplification cascade of proenzyme-enzyme transformations. Contact with surfaces that activate XII promotes fibrinolytic activity in plasma (Soulier & Pron-Wartelle 1959) which does not occur in Hageman trait plasma. It appears that XIIa probably acts similarly to streptokinase by producing a complex with a plasminogen precursor. Ratnoft (1966) was unable to confirm the activating effect on fibrinolysis although this may have been due to different experimental conditions. The activation of the intrinsic plasma Kinin forming enzymes by glass was described by Armstrong et al., (1954) and Margolis (1957) demonstrated that this was dependent upon XII-XIIa.

Donaldson (1968) reported that active XII can convert C'I to the active C' esterase , a globulin. The activation, but not the activity of C' esterase is inhibited by soybean typsin inhibitor, Heparin, and the proteolytic inhibitor from bovine lung. The results suggest that XII does not activate C'I directly but through triggering the fibrinolytic and Kinin forming reactions in plasma. Plasmin activates C'I (Ratnoft & Naft 1967) as may Kallikrein. This central locus of action is impressive and ensures hypotheses about inhibitors of XIIa; possibly as XII-inhibitor complex, the inhibitor being absorbed onto foreign surfaces in order to activate XII. Unfortunately despite this elegant concept people who are XII deficient are not handicapped in any undue way; they bleed to excess but slightly; and react adequately to injury, microbial attack, immunological challenge or mechanical irritation.

Work performed in a number of laboratories has led recently to the recognition of the alternative pathway of complement activation; operative in the absence of immune antibodies and of the early acting

components C1, C2, C4 (Gewurz, Shin & Nergenhagen 1968). These observations are now linked to the properdin system. This can be defined as that group of proteins which in the absence of classical C components C1, C2 and C4 will mediate the proteolytic activation of C3 and C5 when serum is incubated with various polysaccharides and lipopolysaccharides. These substances include endotoxins, yeast cell walls, agar, dextran and polyanionic substances. The activation of C3 and C5 results in the generation of biological activities associated with the small fragments of C3 and C5 (anaphylatoxins C3a and C5a) the chemotactic C5b67 complex and potentially cytolytic C5b6789 complex.

The polysaccharides and polyanionic substances (for example, dextran) which can activate the alternative pathway are also anti-coagulants. The alternative pathway is dependent on this activation of C3 (Figure II). There is dependence on a positive feedback mechanism through the requirement of C3b for the activation of C3PA to C3 a and the efficient clearage of C3 by C3A. Thus a product of the reaction is not only indispensable as a co-factor for the activation of the enzyme which drives the reaction, but is also vital for the effective functioning of this. These functions of C3b are controlled by C3bINA which cleaves C3b into 2 inactive fragments C3c and C3d.

The biological importance of the C3b dependent feedback mechanism is illustrated by the fact that C3b regardless of the mode of formation can switch on the system and recruit the biological activities associated with activation of C3-C9. Trypsin, thrombin, plasmin and lysosomal proteases can cleave C3, which emphasises the

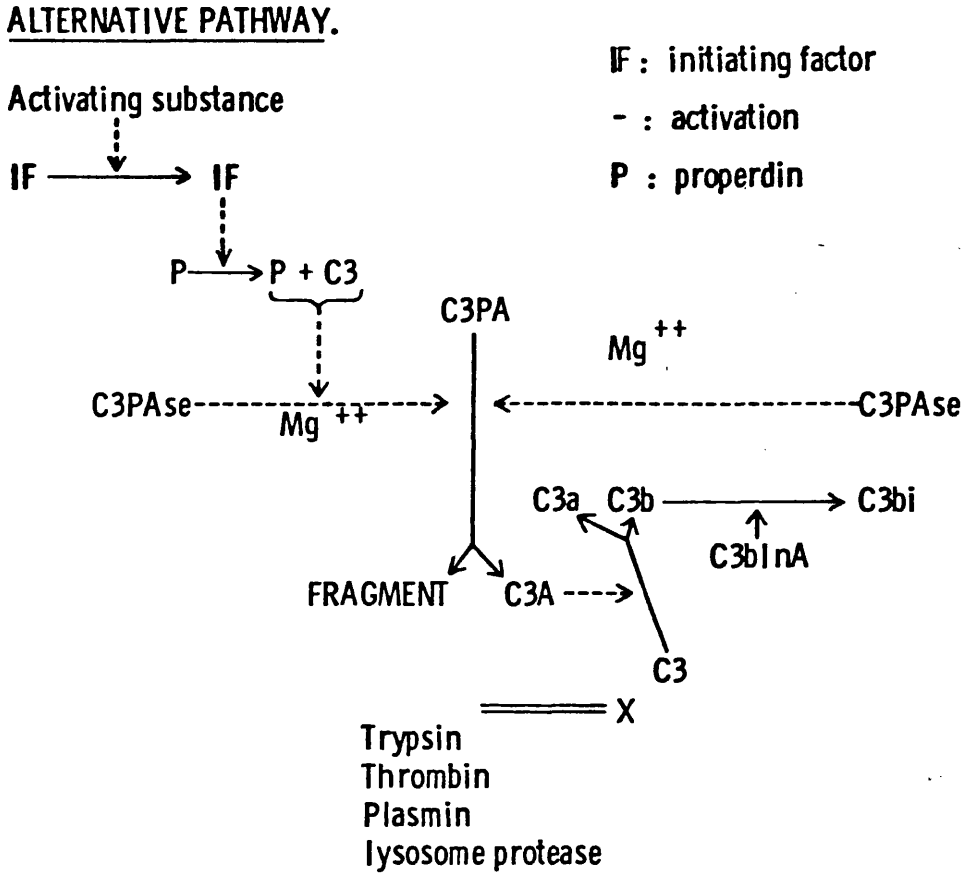

ALTERNATIVE PATHWAY.
IF : initiating factor
- : activation
P : properdin
Activating substance
IF
IF
P
P + C3
C3PA
Mg ++
C3PAse
Mg ++
C3PAse
C3a
C3b
C3bi
C3bInA
FRAGMENT
C3A
C3
X
Trypsin
Thrombin
Plasmin
lysosome protease

Fig. II

confusing interplay of influences involving fibrin and complement.

There remains to consider a further complication to this confused scene: whether plasmin is the only, or even the most important, fibrinolytic agent present in blood. Moroz & Gilmore, employing an exact assay of fibrinolytic activity using I^{125} fibrin absorbed onto polystyrene tubes have shown convincing evidence that spontaneous fibrinolysis is only slightly related to plasmin production, whereas it appears far more related to complement activity. Agents producing complement activation were just as active at enhancing fibrinolysis as were plasminogen activators. It is not surprising that changed fibrinolysis could be thought to have relevance in immunity if fibrinolysis and complement - at least partially - share the same proteolytic cascade.

The last problem in relationship to the thesis is that of tissue plasminogen activators and tissue proteases. Macrophages and polymorphonuclear leucocytes are capable of being stimulated to release plasminogen activator. For example, Unkeless, Gordon & Reich (1974) found that following the intraperitoneal injection of thioglycollate (an irritant) the ability to lyse fibrin was increased a hundred fold. A similar phenomenon was found following the injection of BCG. It has been suggested that enhanced proteolysis may provoke neoplastic transformation. Most malignant cell lines derived by transformation using either oncogenic viruses or chemical carcinogens demonstrate significantly increased levels of plasminogen activator than when compared to their normal counterpart, (Unkeless et al., 1973).

An early event associated with malignant transformation is the production of plasminogen activator (Rifkin & Bader, cited by Christman, Acs, Silagi & Silverstein 1978): tumourigenicity and plasminogen activator disappear concurrently when mouse melanoma cells are grown in BrdU, reappearing when the drug is removed; trypsin applied to cell free nuclear fragments increase the activity of DNA polymerase and DNA methylation. Plasminogen activator within transformed cells could serve a regulatory function whereas the production of plasmin in the surrounding medium affords the neoplastic cell a mechanism for changing its chemical milieu: proteases release chick embryo fibroblasts from contact inhibition, stimulating cell division and deoxyglucose transport (Sefton & Rubin 1970).

There appeared, therefore, to be sufficient evidence to suggest a possible connection between immunity and the fibrin equilibrium. If this were better defined it might be possible to alter the immune response by manipulation of the equilibrium. This would of course not only be of interest in the field of neoplasia, but also in other areas, especially where abnormalities of the fibrin equilibrium occur: transplant rejection, glomerulo nephritis.

Obviously, there are problems associated with the known experimental findings: dampened delayed hypersensitivity due to Warfarin or Heparin; possible neoplastic risk with plasmin inducers. These limit clinical application at the moment.

The aim of this work was therefore to explore the possibility of an alteration in immunity due to changed fibrin equilibrium employing two common tests of in vitro cellular immunity: the

leucocyte migration (LMT) and the ability of lymphocytes to incorporate thymidine during transformation to lymphoblasts. I then used these to determine the effects of three classes of drugs: anticoagulants, such as Warfarin and Heparin; protease fibrinolytic agents streptokinase and plasmin and the fibrinolytic antagonists epsilon amino caproic acid (EACA) and Aprotinin.

I shall describe these tests of in vitro cellular immunity, their problems and their validity in the following chapter.

CHAPTER 2

LEUCOCYTE MIGRATION TEST (LMT)

In the late 19th century it was realised that leucocytes were involved in the inflammatory response, Leber in 1888 thought that a bacterial product "Phlogosin" was released from an area of inflammation which was chemotactic for leucocytes. Massart & Bardet in 1891 suggested that leucocytes were a source of endogenous chemotactic agents.

The development of an in vitro test employing these ideas rests on the descriptions by Rich & Lewis in 1932 of antigen induced inhibition of cell migration from sensitised tissue explants in vitro. George & Vaughan (1962) utilised this by packing guinea pig peritoneal macrophages into capillary tubes. They studied the migration of the cells from the tubes and the inhibition of migration to antigen (particularly PPD). This gave a versatile test which was quantitative, and was found to be a reliable test of cellular immunity in the guinea pig.

Carpenter (1963) showed that the migrating cells inhibited by antigen was the macrophage. Taking advantage of the fact that peritoneal exudates of guinea pigs are about 75% macrophages and 15% lymphocytes, Bloom & Bennett in 1966 separated these cell types, and showed that antigen induced inhibition was dependant upon a mixture of both lymphocytes and macrophages. Macrophages of sensitised animals were not inhibited by antigen alone. This demonstrated that an intermediate step involving lymphocyte antigen reaction was necessary with the production of a soluble product. This was confirmed by allowing normal exudate cells to migrate in chambers

filled with cell free supernatants of sensitised lymphocytes. Clear inhibition of migration was obtained. Identical results were obtained by David (1966) when studying the migration of normal peritoneal exudate cells.

These findings, important for immunological theology, became expanded by the idea that antigen-lymphocyte reaction could produce a wide array of products biologically active but not antibodies (termed lymphokines by Dumonde et al 1969). Many phenomena have been described in association with activated lymphocytes - the table lists only some of the lymphokines that have been described. None have been identified chemically and therefore can only be classed in terms of biological activities. Their names and numbers reflect the assay systems and the differing activities could represent one molecular entity or many. Lymphokine production can be detected early in the activation cycle (GI phase). It stops when the cell starts to prepare to divide by replicating nucleic acids (S phase) when blastic transformation occurs. Leucocyte inhibition factor (LIF) is the lymphokine detected in the leucocyte migration test (LMT) first described by Søborg & Bendixen (1967). By using peripheral blood leucocytes packed into a capillary tube and comparing the migration into medium or antigen they could detect leucocyte inhibition. They were able to demonstrate correlation between the results of skin testing and leucocyte inhibition to brucella antigen and later applied the test to a variety of clinical situations (Bendixen 1967;1968;1969). Subsequently the LMT has been used extensively as an in vitro test of cell mediated immunity, but despite this there is no internationally agreed method, nor reference standards.

LYMPHOKINES

1. Migration Inhibitory Factors

(a) Antigen-Independent Macrophage Migration Inhibitory Factor	MIF
(b) Antigen-Specific Macrophage Migration Inhibitory Factor	SMIF
(c) Buffy coat Leucocyte Migration Inhibitory Factor	LIF

2. Factors Stimulating Macrophage Function

(a) Macrophage Activating Factor	MAF
(b) Specific Macrophage Arming Factor	SMAF
(c) Pinocytosis Activating Factor	PAF

3. Chemotactic Factors

(a) Chemotactic Factor for Macrophages	CFM
(b) Chemotactic Factor for Lymphocytes	CFL
(c) Chemotactic Factor for PMN Leucocytes	CFP

4. Mitogen Factors

(a) Antigen Independent Mitogenic Factor	MF
(b) Lymphocyte Transforming Factor	LTF
(c) Antigen-Dependent B Lymphocyte Mitogenic Factor	LMF

5. Cytostatic Factors

(a) Lymphotoxin	LT
(b) Proliferation Inhibitory Factor	PIF
(c) Colony Inhibitory Factor	CIF

6. Factors Stimulating B Lymphocyte Maturation and Antibody Production (Thymus Replacing Factors)

(a) Specific Factor	'IgT'
(b) Non-Specific Factor	NSF
(c) Allogenic T cell Replacing Factor	TRF

7. Other Factors

(a) Skin Reactive Factor	SRF
(b) Interferon	IF

The problems to overcome before the LMT could be used as a pharmacological tool was to define areas in the methodology of high variability, and if possible to reduce these: and to ensure adequate validation of this method as an in vitro model of delayed hypersensitivity. I shall firstly describe the routine method which I inherited from Mr. Richard Wood, and then analyse each part of this test. I shall then describe validation techniques.

Method

Venous blood was obtained from a subject between 8.30 and 10.00am, without using a tourniquet. 20mls were placed into a sterile universal container containing 250 iu of preservative free Heparin (Weddel) a little of which had been drawn into the syringe prior to use.

The blood was sedimented by mixing with Plasmagel (Laboratories Roger Bellon) to the proportions 1:2 Plasmagel to blood. The blood Plasmagel mixture was allowed to stand for 20 minutes whereupon the white cell rich plasma was removed using a pasteur pipette.

The cells were then centrifuged (5 minutes at 100G) and washed x3 in phosphate buffered saline (PBS).

They were then resuspended in Eagles medium (Wellcome) containing 10% foetal calf serum (FCS) to a concentration of $5x10^6$/ml. By capillary traction the cells were drawn into capillary tubes (Hawksley micro haematocrit) and one end sealed with plasticine. The tubes were centrifuged at 100G for 5 minutes and broken at the cell-fluid interface.

The prepared capillaries were placed into migration wells (Sterlin) two to each well, using a small blob of stopcock grease at the periphery of the well to hold the capillary tubes. The well was then filled with medium containing 10% FCS (for controls) or medium x10% FCS containing antigen (PPD, Ministry of Agriculture, Weybridge). The wells were covered with glass coverslips placed onto previously greased well walls and incubated at 37°C in a CO_2 enriched incubator for 18 hours. The areas migrated were measured by using a microscope with a camera lucid attachment. Migration indices obtained being:

$$\frac{\text{(a) the area migrated in the presence of antigen}}{\text{(b) area migrated in medium}}$$

Statistics

Differences between (a) the area migrated in antigen and (b) the area migrated in medium were assessed by the Mann Whitney Wilcoxon 'U' test. If control areas were not different Student's 't' test was used to compare migration indices, however in tests on different subjects with different control area Feiller's theorem (Finney 1964) was used which assumes the index is a ratio of two normally distributed variables.

Analysis of Method

1. Anticoagulation

Other anticoagulant methods chosen were (1) EDTA and (2) Defibrination. EDTA anticoagulation appeared to produce cytotoxicity: mean migration areas (without antigen) were low (20mm) and PPD in

high concentration (300mgms/ml) produced insignificant inhibition. Trypan blue dye exclusion studies confirmed the cytotoxicity.

Defibrination was performed by shaking venous blood for 15 minutes in the presence of paper clips. Two problems occurred with this technique: an increased red cell contamination of the leucocyte enriched plasma and a tendency for platelet leucocyte clumping to occur when the cells were washed. Despite this the results were similar to those obtained with the standard technique (Table 1).

2. Method of Sedimentation

Blood anticoagulated with Heparin was sedimented for 20 minutes either alone, or mixed with Plasmagel. The resulting leucocyte enriched plasma samples were compared using the standard technique. There was no significant difference in control areas, or in inhibition areas to PPD. Greater cell numbers were obtained using Plasmagel separation and this separation method was therefore used.

Both the defibrinated technique of anticoagulation and the Plasmagel separation produce significant red cell contamination. Despite this there had been no significant effect on migration inhibition: a fact which was at variance to some early work (Falk et al 1970). I therefore used leucocyte enriched plasma produced by separation with Plasmagel to test the effect of red cell depletion (using osmotic shock) and red cell contamination (adding known concentrations of red cells). Osmotic shock depleted red cells, but significantly diminished ($p < .05$) inhibition to PPD in three experiments on a Heaf 1 + subject. This was due to leucocyte cytotoxicity (assessed by trypan blue exclusion). Adding red cells

TABLE 1

Comparison of standard technique using PPD 150 µg/ml Heparin anticoagulant with defibrination anticoagulation, Means ± ISD.
Migration Indices.

Heaf grade	Heparin	Defibrination	Number of Patients
0	0.94 ± 0.12	0.96 ± 0.14	5
1	0.79 ± 0.09	0.77 ± 0.12	3
2	0.76 ± 0.096	0.75 ± 0.15	3
3	0.56 ± 0.25	0.53 ± 0.34	3

to produce a concentration of red cells of $1x10^5$/ml produced no alteration of migration inhibition. This degree of contamination with red cells was greater than seen with any of the methods I used to prepare the leucocyte enriched plasma. Minimal red cell contamination could be safely ignored.

Sedimentation and Aspiration Techniques

The standard sedimentation technique was compared with allowing the Plasmagel blood mixture to sediment for 40 minutes. There was no significant difference observed in migration inhibition, neither was there a significant difference in cell numbers obtained. This excluded the removal of the lower 2mm of buffy coat. When the buffy coat was completely removed without significantly increasing red cell contamination, cell clumping frequently occurred during centrifugation. Three experiments were performed, which although showing no difference in mean migration inhibition or control areas, showed that platelet contamination significantly ($p < .05$) increased variation with a high standard error (Heaf 1 + platelet poor migration index 0.78 ± 0.095, platelet rich 0.75 ± 0.29). This problem with platelet contamination was significantly reduced, if instead of cutting the capillary tube at the cell fluid interface it was cut just below the interface (c 0.5mm) which 'lost' the platelet plug. This procedure, cutting below the cell fluid interface, was adopted in the standard technique.

Washing Technique

One technical problem which I have mentioned is that of platelet-leucocyte clumping during the cell washes. Microscopy of these 'clumps' revealed that they consist of platelets and leucocytes.

Clumping could be provoked if the standard centrifuging times and speeds were used but Calcium Chloride was added to the PBS. This suggested that the problem was produced by platelet clumping (Ca^{++} dependent) occurring when platelets are damaged by centrifugation. Using a platelet enriched, leucocyte deplete plasma, pure platelet clumps could be induced. If leucocytes were then added platelet leucocyte clumping occurred. Various centrifugation techniques were tested, but the standard procedure appeared the most satisfactory compromise and clumping occurred rarely provided that following centrifugation, medium was aspirated quickly and the cells resuspended immediately. A useful technique was developed for use with defibrinated blood (which predisposed to clumping): leucocyte enriched plasma obtained by Plasmagel separation was initially centrifuged at 50G for ten minutes. This allowed separation of platelets from leucocytes, but involved the loss of significant numbers of leucocytes. Following this stage further washes were performed conventionally, without cell clumping developing.

The number of cell washes is obviously arbitrary. It is however difficult to ensure complete separation of cells from contamination with autologous plasma. Techniques to improve this involve the use of different centrifuge tubes and pasteur pipettes for each wash therefore discarding plasma proteins adherent to the glass ware. Since an increased number of washes (x6) significantly decrease migration inhibition to PPD ($p < .05$). To see if this was due to increased loss of autologous plasma, I compared the migration inhibition achieved following the standard 3 washes with that of 6 washes, and to that following 6 washes to which 0.1% autologous plasma was re-added. Whilst there was diminished inhibition following 6

wahses when compared with the standard 3 this was not changed when autologous plasma was re-added. I confirmed by dye exclusion, that the increased number of washes led to cell death.

Three washes were therefore used as a suitable compromise but with the use of clean centrifuge tubes and pipettes at each stage.

Effect of Serum Supplementation of Medium

Others had noticed disconcerting changes of migration inhibition with serum of different species or even batches of serum from the same species. Migrations were therefore attempted in the absence of serum supplementation, but were clearly unsatisfactory: migration areas were small ($<$ 10mm controls) and significant inhibition did not occur even in the presence of high concentrations of PPD in strongly Heaf positive individuals. I therefore compared the effect of various concentrations of foetal calf serum (Boroughs Wellcome, Batch No. K1903) ranging from 5-40%. All the concentrations gave statistically similar results, except that variability with 5% supplementation was significantly greater than in the higher concentrations. It therefore appeared that 10% supplementation was the most reasonable concentration in terms of results and cost.

Capillary Well Interface

If the capillary tube does not contact the migration well floor then the migration area appears to be low. By comparing control areas using the standard technique with that where the capillary had been purposively left contacting the well floor, I demonstrated reduced migration in the latter group ($p < .01$). This was one of the most obvious causes of error in the technique.

pH of Medium

Most observers had found the migration inhibition to be pH dependent, which I confirmed. A pH of culture medium less than 7.0 resulted in lost inhibition to antigen although control areas were identical to those where the pH was 7.2. The pH was always monitored therefore, both by the use of pH labile dye in the Eagles medium, and by measurement of the pH prior to the addition of culture medium to the wells.

Time of Blood Storage

Sometimes it was necessary to store blood samples for up to 4 hours before testing. Clearly it was important to assess the effect of storage in the assay. Blood was therefore taken from two volunteers_ migration inhibition assay performed immediately or 4 hours storage at room temperature. There was no significant difference in either control, or inhibition areas.

Leucocyte Cell Numbers

Leucocytes were prepared to the concentration of $1X10^6$/ml in the standard technique, but the actual cell numbers in the capillaries deviated from this by about 15%. This could be simply assessed by measurement of the length of cell column following centrifugation. The variation was dependent upon cell sedimentation prior to insertion into the capillaries (which could be decreased by resuspension by shaking at minute intervals). An experiment to assess the importance of this variation was therefore performed altering the numbers of leucocytes from $1x10^5$ to $1x10^7$/ml. Migration

control and inhibited areas were not statistically different at these wide concentration limits. This did not therefore appear to be source of error, although concentrations were kept at $1x10^6$/ml for the standard technique.

Migration Time

Clausen (1973) in a perceptive critique of migration inhibition showed that the inhibition of migration due to PPD was time dependent: mantoux negative and mantoux positive group differed significantly at 4 hours ($p < .001$) but not at 20 hours ($p < 0.1$). However this was employing glass migration wells: using polystyrene wells the 20 hour migration inhibition was similar to the 4 hour values. At each time point the migration indices were significantly lower than when using glass wells (in mantoux positive patients). In fact he demonstrated that the results of the LMT using polystyrene wells were in close agreement to the agarose technique of migration inhibition.

Time course studies were performed to ensure that the 20 hour migration inhibition was satisfactory even though since I was using polystyrene wells this appeared likely. Migration indices were compared at 1, 4, 18 and 20 hours in a group of subjects of varying Heaf reactiveness. The results are shown in Table 2. Using Feilers theorem, there was no significant difference between indices measured at 4, 18 and 20 hours. Of great interest was the relative stimulation achieved at 1 hour in the Heaf positive grades. As a group this was not significant, but in individual cases, there was a significant stimulation (determined by 'U' test). Some of the results at 1 hour therefore are at conflict with the concept of leucocyte inhibition, but

TABLE 2

Migration Inhibition to PPD : Time Course of Migration Inhibition
(PPD 200 ug/ml)

Heaf Grade	Number	Migration Indices ± ISD 1 hour	2 hour	18 hour	20 hour
0	4	1.05 ± 0.10	0.98 ± 0.15	0.94 ± 0.15	0.96 ± 0.12
1	3	1.15 ± 0.25	0.795± 0.15	0.78 ± 0.11	0.79 ± 0.09
2	3	1.05 ± 0.28	0.75 ± 0.17	0.77 ± 0.10	0.76 ± 0.096
3	3	1.21 ± 0.36	0.63 ± 0.21	0.61 ± 0.22	0.59 ± 0.25

PPD is only added to the medium in the well. Since the cells in the capillaries are packed tightly following centrifugation it is reasonable to speculate that initially there is an antigen gradient.

Under these conditions leucocyte chemotaxis would occur towards the higher concentration of antigen. To test this idea, migrations were performed, with measurement at 1 hour using PPD (200 ug/ml) in the migration well in the standard technique; in the cell suspension prior to insertion into the capillary tube as well as in the migration well; and in the third group only in the cell suspension packed into the capillary tube. Three Heaf positive subjects were tested: the mean migration index for the standard technique at 1 hour was 1.10 ± 0.18 (ISD); for the PPD present in cells and medium 0.82 ± 0.21 and for the group with PPD present only in the cell mixture 0.65 ± 0.17. These results were significantly different from each other ($p < .01$) and supports the concept of interference at 1 hour from chemotaxis; thus the group with antigen present at equal concentrations in both medium and cell suspension exhibited purely migration inhibition; those with antigen present only in the cell suspension exhibited an additive effect of migration inhibition and chemotaxis; whereas those with antigen only in the medium in the well exhibited a conflict between chemotaxis and migration inhibition. When the migrations were reviewed at 4 hours and 20 hours there was no statistical difference between the standard technique and that where antigen was added both to the cells and to the medium in the well, but at these times no significant inhibition was observed where the PPD was added only to the cell suspension.

I believe that this hypothesis could explain the occasional significantly stimulated values seen: it is reasonable to suppose that leucocyte chemotaxis might occur at a different antigen concentration to migration inhibition; certainly however for routine purposes there is no value to be gained from the more complicated addition of antigen to the cell suspension.

Although values at 4 and 20 hours are comparable, statistical analysis of this depends upon Feiller's theorem since the control areas are different. Obviously this is a relatively insensitive statistical test and therefore for a combination of convenience and accuracy 20 hours was adopted as standard. Later the use of 4 hours migration indices are important in the multistage LIF assay.

Dose Response to PPD in the LMT

4 Heaf negative subjects and 4 Heaf 1 positive subjects were studied. LMT were performed using varying concentrations of PPD. The results are shown in Table 3 .

These suggested a likely concentration of toxicity occurring to PPD at about 400 ug/ml because of the significant fall in migration inhibition at this level. The dose response curve to PPD in the Heaf positive group was complex: significant inhibition then followed a log response to 300 ug/ml but at 400 ug/ml there was again a rapid impairment of migration: suggesting cytotoxicity. This conclusion was confirmed by the results of dye exclusion viability studies. At 400 ug/ml cell death had reached highly significant levels.

TABLE 3 Dose Response to PPD in 4 Heaf Negative and 4 Heaf Positive Subjects Mean ± ISD

Heaf Grade	Subject	Control area mm^2	Area with PPD 50/ug/ml mm^2	PPD 100 ug/ml mm^2	PPD 150 ug/ml mm^2	PPD 200 ug/ml mm^2	PPD 300 ug/ml mm^2	PPD 400 ug/ml mm^2
0	A	115 ± 6	124 ± 9	113 ± 7	119 ± 8	111 ± 7	110 ± 6	105 ± 7
0	B	122 ± 8	129 ± 7	131 ± 9	125 ± 7	119 ± 11	121 ± 9	115 ± 7
0	C	118 ± 5	124 ± 4	116 ± 12	120 ± 7	117 ± 9	118 ± 6	92 ± 7
0	D	120 ± 9	115 ± 8	126 ± 7	123 ± 9	119 ± 11	114 ± 7	82 ± 14
1+	E	117 ± 7	123 ± 11	125 ± 9	92 ± 4	84 ± 5	78 ± 7	67 ± 21
1+	F	119 ± 10	128 ± 6	102 ± 12	93 ± 7	88 ± 7	80 ± 9	63 ± 17
1+	G	116 ± 4	124 ± 12	117 ± 9	88 ± 8	82 ± 8	79 ± 11	62 ± 12
1+	H	123 ± 8	119 ± 6	121 ± 5	97 ± 9	89 ± 7	83 ± 13	55 ± 18

Fpr population studies I therefore chose 150 ug/ml PPD, since this gave a convenient separation between Heaf negative subjects and Heaf positive, but for assessment of drug-antigen cytotoxicity I chose the highest non toxic concentration of PPD (300 ug/ml).

Number of Capillary Tubes in Each Well

Obviously using 2 capillaries in each well reduces the amount of antigen required, it was therefore part of the standard technique and was used in the early experiments on epsilon amino caproic acid, EACA (Burden et al 1978). However, when analysis was performed upon the results obtained using this method with that of one capillary in each well it became apparent that although the variation produced by the 2 capillary technique was low, this was false. The areas were sometimes misread because of overlapping; obviously statistical analysis using the 2 capillary technique should not count each as a separate result, but as a mean; which gives good results but uses many cells.

For later experiments only 1 capillary was therefore used in each well.

Extrinsic Sources of Variation

1. Diurnal Rhythm

Despite the wide use of LMT as a clinical in vitro assessment of cellular immunity, there is surprisingly little documentation of the possibility of a diurnal rhythm. There are of course acceptable reasons for supposing that a diurnal rhythm could occur; steroids such as hydrocortisone are known to interfere with delayed hypersensitivity, and are themselves subject to wide fluctuations throughout the day.

A further important diurnal variation is in the peripheral blood leucocyte count (Sharp 1960). The only evaluation of the problem was by Richens et al (1975) who analysed the variation in 7 human volunteers and in 15 rats and compared these with total leucocyte count and cortisol concentrations. They discovered a marked diurnal variation in migration area, but surprisingly not in migration inhibition (to DNCB coupled to human serum albumen). The nadir of migration occurred at midnight.

My own studies were on 3 volunteers who were Heaf positive, and 3 patients who had Addison's disease and were being studied for other reasons (Clayton et al 1977) Their Addison's disease was secondary to adrenal tuberculosis. Migration inhibition to 150 ug/ml PPD was performed on the 3 volunteers at 800 hours, 1200 hours, 1400 hours and 2000 hours. The results are demonstrated in Tables 4&5. Clearly there was no significant change in migration index, but the control area was significantly lower at 2000 hours: confirming the work of Richens et al. Of equal importance was the lack of significant change during the normal working day, and although it is preferable to perform the migrations at a uniform time the results are comparable when bloods are taken between 8am and 2pm.

The 3 patients with Addison's disease underwent for other reasons the following protocol: on day 0 they omitted their usual steroid dosage in the evening, day 1 they omitted their usual steroids for the whole day, and on day 2 they were admitted, basal bloods taken at 8am for cortisol levels and for migration inhibition. They were then given 30mgms of hydrocortisone orally and bloods taken for cortisol levels at 30 minute intervals. At 4 hours (1pm) and 8 hours (5pm)

TABLE 4 Diurnal rhythm : LMT in 3 volunteers (Heaf +ve)

Time (Hours)	Control Area Mean ± ISD	Migration Index ± ISD
800	126 ± 5 mm	0.77 ± 0.11
1200	132 ± 5 mm	0.80 ± 0.12
1400	127 ± 9 mm	0.76 ± 0.10
2000	97 ± 11mm	0.79 ± 0.15

further LMT's were performed. The results are shown in Table 5 . Obviously these results are limited, but important. The basal values were taken when there was virtually no cortisol circulating (the value 34 mol/L approaches the limits of assay). Since hydrocortisone has a half life (circulating) of about 1-2 hours (Migeon et al 1956) this state would have existed for about 24 hours previously. Both migration area (control) and migration inhibition to PPD were significantly lower than that seen following the hydrocortisone. This suggested that whereas high concentrations of a glucocorticoid impair delayed hypersensitivity reactions inordinately low levels (probably associated with a host of intracellular biochemical abnormalities) also impair delayed hypersensitivity. It also confirms the findings of Stevenson (1973) who demonstrated that addition of cortisol to the medium into which the cells migration stimulates polymorph migration in vitro. Despite a markedly significant fall in cortisol levels between 4 and 8 hours following oral hydrocortisone ($p < .01$) there was no significant change in migration area or inhibition. This is probably related to the prolonged biological half life effect seen with steroids; and which helps to account for the useful finding that migration areas and indices are similar during the day.

These results have since been confirmed by studying a 48 year old man, thought on clinical grounds to have mild Addison's disease (fatigue, lassitude, mild hyperpigmentation, normal electrolytes). Together with diagnostic tests for his Addison's disease (9am and midnight cortisols and ACTH, synacthen test) a Heaf test was performed and a LMT to 200 ug/ml of PPD. The Heaf was negative at 48 hours and

TABLE 5 3 Patients with Secondary Addison's Disease : Cortisols and Migration Inhibition Following 24 hours without Replacement, and 30mgms Hydrocortisone orally at 8.30h

Time (hours)	Plasma Cortisol nmol/L	Migration Area Mean ± ISD	M.I. Mean ± ISD
800	34 ± 9	62 ± 19	0.19±0.14
1300	560 ± 125	138 ± 11	0.64±0.12
1700	255 ± 205	136 ± 15	0.65±0.10

the LMT showed no significant inhibition. The clinical diagnosis was confirmed and he was started on hydrocortisone at 72 hours: within 24 hours he developed marked positivity of his previous Heaf (2+) and the LMT became significantly inhibited (MI 0.68 $p < .05$ using 'U' test).

To summarise: there is a marked diurnal rhythm of the LMT, probably related to cortisol levels, but this is not of importance if the blood is taken between 8am and 2pm.

Variation with Time

Two volunteers weakly Heaf positive (1+) had LMT performed weekly for 2 months using PPD 150 ug/ml. The results were analysed using Feiller's theorem, and showed no significant variation, except on one occasion in one subject 24 hours after developing the symptoms of a common cold. The LMT therefore gives consistent results in normal healthy subjects over a 2 month time period. Any excessive variation may be likely to be due to concurrent illness.

Correlation With Other Tests of Cellular Hypersensitivity

The informed consent was obtained for 29 people, 21 patients and 8 normal volunteers. Heaf tests were performed, responses measured and individuals graded 0 to 111+ using the criteria established by Pagel et al (1964). The results identified a group of 4 patients with carcinomatosis previously known to react to PPD but now anergic.

The standard LMT was used as outlined above. The results are shown in Table 6 . Clearly there is a reasonable correlation between the skin test, and the LMT justifying its use as an in vitro assessment of delayed hypersensitivity.

TABLE 6 Correlation of Heaf and LMT in 21 patients and 8 volunteers

Heaf Grade	Number of Patients	MI Mean ± ISD
0	6	0.95 ± 0.125
0 previously +ve	4	0.98 ± 0.1
1+	4	0.78 ± 0.085
2+	3	0.76 ± 0.096
3+	6	0.61 ± 0.26
Miliary Tubercle	6	0.56 ± 0.15

Two Stage Leucocyte Migration Test

The LMT has been shown to correlate with another index of cellular immunity; the Heaf test. It is simple and cheap and the inherent variability can be reduced by close attention to detail. In the standard LMT the lymphocytes obtained from the venous blood are believed to produce the lymphokine LIF which simultaneously inhibits the migration of PMNL. To examine an alteration in this test seemed reasonable to divide the standard LMT into three parts: lymphokine generation: lymphokine stability in medium: and lymphokine assay.

Material and Method

The culture medium used was Eagles MEM containing antibiotics and 10% foetal calf serum, pH 7.2 PPD (Weybridge) was used in a working concentration of 150 ug/ml.

(1) Cell separation. To produce relatively pure populations of either lymphocytes or polymorphonuclear leucocytes, blood anticoagulated with Heparin was sedimented with Plasmagel (15 minutes at room temperature). The buffy coat was removed and carefully layered onto ficoll/hypaque. Following centrifugation (20 minutes at 250G) the lymphocytes layer or the PMNL could be collected separately. These cells were then washed three times in phosphate buffered saline and resuspended in medium. If either of the cell suspensions showed more than 5% contamination with the other cell type (as shown by staining and examination with light microscopy) the experiment was abandoned.

(2)(a)Lymphokine generation. Lymphokine containing supernatants were generated in a PPD-lymphocyte culture either for 4 or 24 hour periods.

Lymphocytes, at a concentration of $1x10^5$/ml were incubated with PPD at 37°C, or for control cultures in medium alone. At the end of the culture period the cells were spun down and supernatants obtained. PPD was then added to the control supernatant.

(2)(b)Lymphokine stability. This lymphokine containing supernatant could then be either assayed immediately, or following an intermediate incubation, at 37°C, for either 4 or 24 hours. Thus stability of the lymphokine LIF in medium could be determined.

(3) LIF Assay. Fresh PMNL suspended in medium with serum were placed into capillaries the ends of which were sealed with plastercine and the capillaries then centrifuged for 5 minutes at 150G. The capillaries were cut just below the cell fluid interface and inserted one into each plastic well using a minimum of 6 capillaries for each experimental group. The supernatant to be tested was added to the well. The wells were then incubated for 18 hours at 37°C, the migration areas drawn using a camera lucida microscope attachment and measured by planimetry.

Validation

It was assumed that if a reasonable agreement between this two stage procedure and the conventional single stage could be demonstrated this would be sufficient validation.

Three separate assays were performed in each of 6 subjects. The results are shown in Table 7 . This not only shows a close agreement between the two methods but also good reproducibility of the 2 stage technique.

I then determined the effect of intermediate incubation of LIF containing supernatants. The results are shown in Table 8 which shows that whereas immediate assay and assay after 4 hours intermediate incubation were similar, the 24 hour extended incubation results showed a highly significant loss of activity. Control areas were not affected, therefore this could only be due to loss of LIF or LIF activity.

These results showed that this 2 stage LMT was an acceptable test for investigating the site of action of the various drugs I wished to examine.

TABLE 7 Reproducibility of 2 Stage Method, and Comparison with 1 Stage Technique

Subject	2 Stage: MI ± ISD	1 Stage: MI ± ISD
1	0.76 ± 0.11	0.79 ± 0.07
2	0.69 ± 0.07	0.63 ± 0.05
3	0.54 ± 0.09	0.51 ± 0.08
4	0.65 ± 0.13	0.58 ± 0.07
5	0.73 ± 0.12	0.72 ± 0.06
6	0.74 ± 0.08	0.70 ± 0.09

TABLE 8 The Effect of Intermediate Incubation of LIF Containing Supernatants: 12 Experiments, 2 for Each Subject

Intermediate Incubation Time	Migration Index Mean ± ISD	Significance
0	0.66 ± 0.12	
4 h	0.70 ± 0.10	NS
24 h	0.84 ± 0.09	$p < .01$

LYMPHOCYTE TRANSFORMATION

Yoffey, my Professor of Anatomy at Bristol, asserted that the small lymphocyte was not effete (Yoffey & Courtice 1956). This was only supported late in his academic career by the realisation by Hungerford et al (1959) and Nowell (1960) that phytohaemagglutinin (PHA) an extract of the Red Kidney Bean, phaseolus vulgaris, could transform lymphocytes to large, active cells. Carstairs (1961) demonstrated that the responding cell was a small lymphocyte.

The concept of a dual immune system was also being developed. Based originally on animal studies removing either the bursa of fabricius (Glick et al 1956) or thymus (Aspinall et al 1963); the idea was expanded by the microscopical appearances of PHA stimulated lymphocytes compared with those stimulated by another mitogen, poke week (PWM). A proportion of those transformed by PWM had a hyperplastic rough endoplasmic reticulum; B cells (Chessin et al 1966); whereas PHA induced only T cell transformation.

The Mitogen

The list of possible mitogens has been enormously expanded, artificially by the systematic examination of various remote plant extracts and more methodically by the examination of the chemical structure of the mitogens. An inexhaustive list is proferred in Table 9 .

The first mitogens to be described were the Lectins. These are sugar-binding, can precipitate polysaccharides and glycoproteins and can agglutinate cells. This suggested that they were binding to cell surface saccharides which was confirmed by Lectin specific

TABLE 9

MITOGENS (after Cunningham et al)

1. Plant Extracts	PHA
	Con covalin A
	Lentil
	PWM
	Favin
	Pea
	Wisteria Floribunda
	Peanut agglutinin
2. Bacterial Product	Lipopolysaccharide
	Staphylococcal enterotoxin B
	PPD
	Streptolysin
3. Antibody reagents	Anti Ig sera
	Anti lymphocyte
	Carbohydrate specific
	Anti 2 macroglobulin
	Anti B2 microglobulin
4. Polymers	Ficol
	Dextran
	Poly A-Poly U
	Poly 1
	Poly 1-Poly C
	Polyethylene sulphate
	Dextran sulphate
	Pentosane sulphate

TABLE 9 Cont'd.

5. Miscellaneous Chemicals	Sodium metaperiodate
	Pharbol esters
	Ca ionophore A 23187
	Metal ions : Zn, Hg, Ni
	Proteolytic enzymes, trypsin
	Papain

inhibition by monosaccharides (Barberg et al 1966). This binding to the saccharide components of glycoproteins and lipids could be simply manipulated, by proteases removing blocking constituents. Goodall et al (1971) showed this with a variety of proteases enhancing the activity of PHA in rabbit lymphocytes.

Activation

Following the interaction with receptors there is probably a resulting alteration of membrane comformity (Tanger et al 1970) leading to early changes in transmembrane potential (Vassar & Culling 1964), ion leakage and transport (Allwood et al 1971), questionable changes in cyclic nucleotides (Sith et al 1971), and later changes in amino acid (Vandem Bergh & Betal 1971) and nucleic acid transport (Park & Good 1972). Subsequently the cell divides.

The extent of activation is dependent upon many modulators; serum factors, FCS (Continho & Moller 1973); red blood cells (Tarnvik 1972; Yachnin et al 1972), PMNL (Luquetti & Janossy 1976), autologous xray irradiated lymphocytes (Yachnin et al 1972), macrophages (1974), other mitogens, either T or B (Ivany 1976). These may facilitate or in some cases inhibit (Scott 1972) lymphocyte responsiveness to PHA.

Clinical Significance of Activation

The problem of the clinical significance of lymphocyte transformation by mitogens has two limitations; that most work has concentrated on PHA which whilst convenient, may not be appropriate and methods until recently were laborious. The tests have been used, however, to investigate many clinical conditions: if poor responsiveness

to PHA occurs with normal T cell numbers, it can be concluded that T cell function is impaired (Fudenberg et al 1971).

Measurement of Activation

Measurement was initially by microscopy and blast cell counts, later the use of radiolabelled (C14 or H3) thymidine became standard, although other markers can be examined, for example L amino acids (Kay 1966), adenyl cyclase stimulation (Winchurch et al 1971) or I^{125} iodouridine (Loquetti & Janossy 1976).

The use of thymidine has become classical but there are problems associated with its use; high concentrations inhibit DNA synthesis by interfering with the synthesis of other deoxyribonucleotides (Hartog et al 1967); high specific activity may produce cell death (used in 'hot pulses') whereas low specific affinity produces little differentiation between control and stimulated cultures; thymidine is quickly broken down by PMNL to thymine and dihydrothymine which are not utilised. The rate limiting step is transport across the membrane (Hirschhorn et al 1971).

B Cell Mitogens

Semanticists insist on polyclonal B cell activation; these mitogens do not read the dictionary of Ig specificities and triggering occurs at other sites than the Ig receptor combining sites; the proliferation is not important for early antibody production and does not affect all B cells. Since the blast transformation is often slight in comparison with the antibody response, ideally the B cell should be in a resting state before detection of a potential B cell mitogen is attempted; especially since some other mitogens are B cell

Theoretical B Cell Responses to Mitogens

	B cell mitogen		
1.	Virginal B Cell →	blast with hyperplastic endoplasmic reticulum antibody synthesis	antibody secreting cells
2.	Virginal B cell →	blast no hyperplasia	no secretion ? other factors needed
3.	Virginal B cell →	no response without other factors	

competitive antagonists (Continho & Moller 1975; Mishell & Dutton 1967).

Many B cell mitogens are polymers (the list of polymer mitogens in the table have all been shown to be B cell mitogens) which was one reason for the idea that they enhanced the production of C3 either directly from proteolysis of C42 or via enhanced macrophage proteolysis. The response may be polyphasic especially with polymers; for example, in a dose response to Ficoll, Continho & Moller (1975) reported that at .001 to .01 ug/ml; 1 ug/ml and 100 ug/ml, peaks of stimulation occurred with no stimulation at .1 and 10 ug/ml.

The probable effects of B cell mitogens in vivo on B cell are shown in the figure: with a wide array of possible effects from marked antibody synthesis and secretion to no obvious effect. Although the best medium for exploration of B cell mitogen structure and receptor analysis is synthetic, with only B cells present, the ideal medium for an attempted analysis of the effect of B cell mitogens in vivo should approximate closely to whole blood. This may provide non-specific amplification mechanisms of great importance in a clinical situation. B-T; T-B cell interaction.

When the idea of a dual immune system became accepted there was also a tendency to assume that the thymus derived T cell was only responsible for cellular immune reactions, and the bursa equivalent derived B cell was only responsible for humoral reactions. Subsets of both systems are now recognised. Identified by analysis of responses to mitogens following suicide stimulation by other mitogens in the presence of high activity thymidine. These subjects can also be demonstrated in vivo; Gronowicz & Continho & Moller 1974

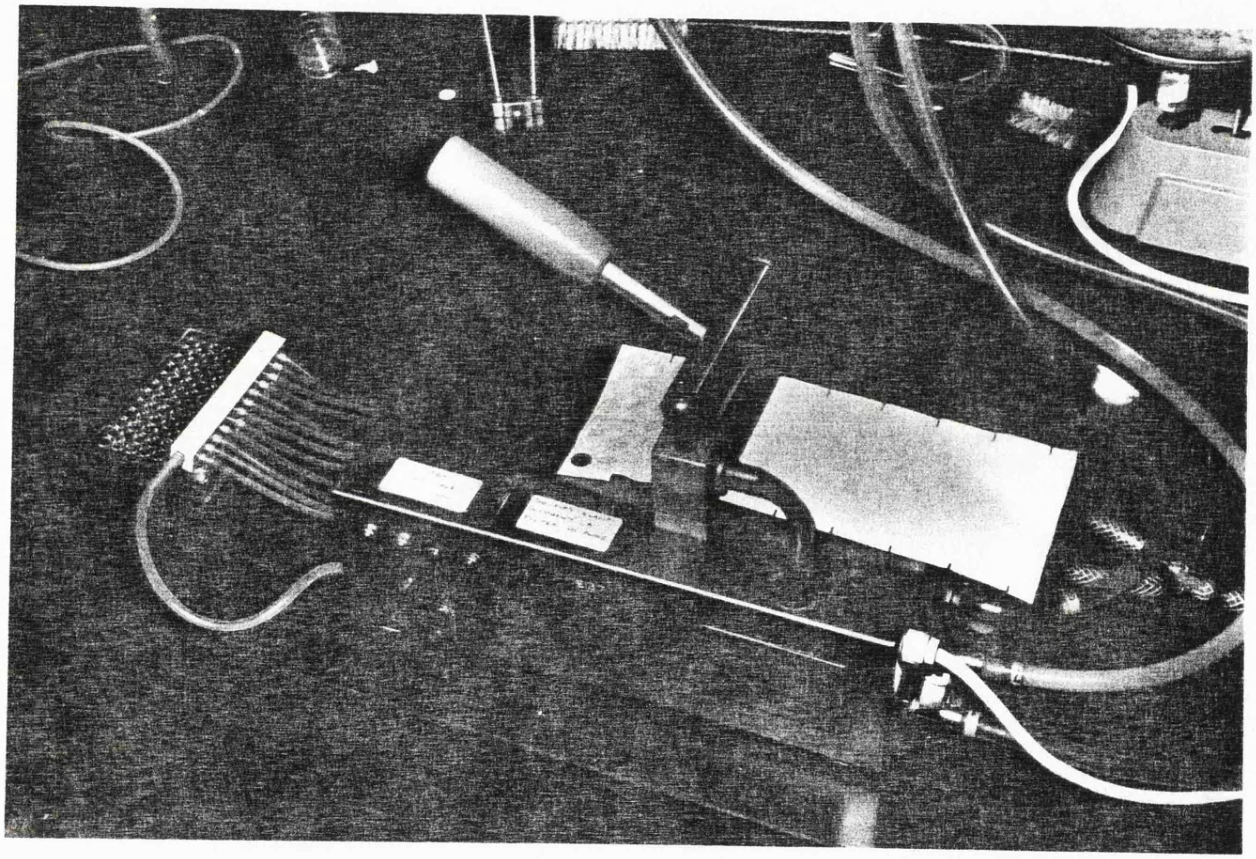
Helper functions as well as suppressor functions are now known to exist in both B-T and T-B cell interactions, which suggest an important physiological role for both T and B cell mitogens.

Methodology

The history of methodology in the measurement of lymphocyte transformation to mitogens has already been suggested. Like many assays it has developed from time and labour consuming assays requiring large volumes of blood to micro techniques. Until recently separated lymphocytes were needed and in the early work activation was detected by light microscopic changes. Separated lymphocytes provoked much work on mediums and supplementation with varieties of serum. With the introduction of thymidine quantification of activation became simpler but initially elaborate procedures were used prior to scintillation. This became simpler with the use of dried filters inserted directly into scintillation insert vials. Although disliked by the purists the use of dilute whole blood rapidly allowed the use of smaller quantities of blood and the development of micro culture techniques. It avoids loss or modification of lymphocytes during the separation procedures and facilitates lymphocyte culture. I decided to use the dilute whole blood technique developed by Luquetti & Janossy. (1976)

Method

Venous blood which was taken between 8am and 12 noon, was anticoagulated with Heparin (preservative free) or be defibrination. It was diluted 1 : 10 in medium TC199; 200 ul volumes were then distributed into microtitre plate wells. At least 16 replicate wells



were used for each drug dilution. The cultures were incubated at 37°C for 48 hours in humidified air with 5% CO_2 added and then labelled for 4 hours with tritrated thymidine (5 ul of 0.5 uCi/ml). Cells were recovered from each well onto filter paper by means of an automatic multiple sample harvester (see picture). The filters were added to the scintillation fluid (PCS) and counted for one minute. The results were quench corrected and expressed as disintegrations per minute (DPM).

Validation

Anticoagulation:

There was no difference between anticoagulation by defibrination or with Heparin either in control or PHA stimulated blood samples (Wellcome purified PHA K 2313 at a concentration of 0.1 ug/ul).

Dilution:

A comparison was made between blood samples from 3 volunteers diluted variously to 1:2, 1:4, 1:10, 1:15 and 1:20 either in control or PHA stimulated incubations. The coefficient of variation was lowest ($>$10%) at the 1:10 dilution although the differences in variation was not significant except at 1:2 and 1:20 dilutions. The mean DPM were highly significantly increased at the lower dilutions, 1:2 and 1:4, but only in the control samples. The results in the PHA samples produced slightly but significantly lower results both at the higher dilution 1:20 and low dilutions 1:2 and 1:4.

In order to accurately control these experiments,further blood samples diluted similarly had not had thymidine added at the conventional time, but rather immediately prior to harvesting. Any thymidine which failed to be washed from the cells adhering to the

filter papers could then be counted. As the results show (Table10) a proportion of the control DPM represents thymidine contamination.

Number of Lymphocytes

Luquetti & Janossy had shown a linear relationship between physiological numbers of T cells and PHA maximum response. Although I reproduced their finding it was of no consequence in the in vitro drug experiments, where blood from the same subject taken at the same time is compared. Similar reasoning applies to B cell numbers.

Incubation Time

Time course experiments were performed, the incubates being harvested at 12, 24, 48 and 72 hours. The time course should be expected to have two characters; initially a slight difference between control and stimulated later with the exhaustion of the medium, lymphocyte death and reduced DPM from stimulated cultures. This was indeed the effect found. The replicates allowed a more accurate assessment of error. Although mean values, stimulated, at 24 hours were less than at 48 hours, since the variation at 24 hours was less, significant differences between stimulated and control values were as often observed at 24 hours as at 48 hours.

Harvester Position

The automatic harvester I used had an inherently poor design feature; inflow was via one tube, see picture. Pressure of the washing fluids was, therefore higher at position 1 than position 16. Careful attention to washing procedure and comparison of the 16 replicates with other replicates reduced this error.

TABLE 10 Comparison of Various Dilutions of Blood : TC199 Upon Thymidine Incorporation (DPM) In Control And PHA Cultures

Dilutions	Control (no thymidine incorporation	Control	PHA
	Mean DPM ± ISE	Mean DPM ± ISE	Mean DPM ± ISE
1 : 2	1867 ± 279	2476 ± 259	80,101 ± 10,160
1 : 4	1603 ± 339	2181 ± 267	86,259 ± 8,231
1: 10	1101 ± 97	1876 ± 173	125,021 ± 5,719
1 : 15	989 ± 103	1719 ± 189	129,021 ± 7,194
1 : 20	897 ± 117	1831 ± 220	94,083 ± 8,124

Carbon Dioxide

Early in the analysis of method there was no facility to conveniently add CO_2 to the incubator. The only abnormality this produced was in the outer wells which demonstrated a pH change, and accompanying evidence of cell death; microscopically and on thymidine uptake. This was circumnavigated by not using these wells. When CO_2 addition became available a further problem occurred, the gas infused was dry and plates dried. A simple tray of water acted as an efficient humidifier, but also bacterial incubator. The final compromise was humidified CO_2 (the water replenished frequently and included an efficient bacteriocidal agent), and semi-permeable cellotape over the plates.

Label Period

A variety of experiments were performed to examine the most efficient labelling period, and quantity of thymidine. Comparing 2, 4, 8 and 12 hour label period (Table 11) the quickest time with a reasonable coefficient of variation was 4 hours. As H3 thymidine can kill cells, a short incubation period is theoretically important, and has obvious practical advantages.

Thymidine Amount

Similar experiments of 4 hour label, varying the quantity of thymidine (5 uls of 0.25; 0.5; 0.75; 1 uCi/ml) demonstrated that the optimum in terms of cost, low variance and large difference between PHA stimulated and control counts was 0.5 uCi/ml. The results of both experiments is shown in the Table.11

TABLE 11 Effect of Time and Quantity of Label (Means ± ISD)

1. Time (hours)	Control DPM Mean ± ISD	PHA Mean ± ISD
2	1213 ± 147	53,259 ± 5,997
4	1903 ± 156	119,340 ± 4,979
8	1790 ± 164	120,112 ± 6,548
12	1887 ± 199	106,248 ± 9,133
2. Quantity Thymidine		
5 uls 0.25 uCi/ml	795 ± 215	44,320 ± 11,259
0.5 uCi/ml	1837 ± 166	130,031 ± 6,121
0.75 uCi/ml	2311 ± 319	136,579 ± 6,950
1 uCi/ml	2749 ± 354	110,678 ± 7,134

Drying Filter Papers

Using the scintillation fluid PCS it was important to determine the best mode of delivery of the harvested cells adherent to the filter; dry or wet; in insert vials or into scintillation vials. There was no significant difference in DPM between dry and (slightly) wet. DPM in insert vials were far higher and more consistent; the filters were of such a size to jam in the insert vial allowing both sides of the filter to be exposed to the scintillation fluid. In scintillation vials the filters tended to stick to the sides and the volume of scintillation fluid was far less in the insert vials. Early experiments (see previously) were performed using scintillation vials, later experiments (Burden et al 1977) insert vials; there was a subsequent change in counts, particularly controls.

Counting Time

For the counting error to be less than 10% about 5000 Cpm are needed, however, the error tends to be reduced by the use of replicates; therefore I adopted a minute count as standard, producing a rather high error on the controls but acceptable for stimulated values. Experiments where drugs with unknown effect were used, were counted until the counting error was less than 10%.

Quenching

Background counts were subtracted, and Cpm converted using a quench correction to DPM. Quenching was approximately 50%.

Expression of Results and Statistics

Lymphocyte transformation, like leucocyte migration inhibition,

is another area of injudicious use of indices. Whereas migration indices can be justified, that of stimulation index can not. It would presume a linear relationship between control and stimulated and that control measurements were related to the number of functioning units. That this is not so in the whole blood method has been shown, but it is also inappropriate in separated lymphocyte methods, primarily for the same reason; control counts tend to be heavily influenced by the excellence of washing. Another reason is that control cultures may have been exposed to mild mitogens (inevitable in separated techniques: Ficoll). The view that the effect of 2 mitogens multiples, is nonsense, and has been shown to be irrational both for T and B mitogens (see for example, Burden et al 1977). Additive, inhibitory or synergistic effects could be predicted and have been seen. The use of stimulation indices should therefore be abandoned. I have reported both control and stimulated values. The distribution of 96 control and 96 (PHA) stimulated incubates were normal and continuous justifying the use of untransformed data in students 't' test.

Reproducibility

As in any assay if any item is changed the results may in the future be altered; even slight changes resulting from a re-calculated quench correction, or worse an altered PHA. Unfortunately there is no easy way to standardise the assay. PHA, of course, has been standardised, and I used the highly purified reduced red cell agglutination type. The problem of reproducibility could be rationalised by the use of cultured lymphocytes or much more cheaply, by demonstrating close reproducibility in a subject over a length of time.

One subject was studied; control and PHA values were compared daily for five days and subsequently monthly for three months. Except at times of intercurrent illness the values were statistically similar.

Validation of Other Techniques

I tested Caproic Acid (EACA) on other techniques of investigating lymphocyte stimulation. A whole blood macro method, separated lymphocyte macro and micro methods were employed. All showed PHA stimulation to a variable extent, and all showed significant reduction of the stimulation by EACA. Dextran sulphate, a known B cell mitogen induced significantly increased counts in both a separated lymphocyte and the whole blood macro technique. This I believe is sufficient validation, although the work on polysaccharides was also validated using a separated lymphocyte micro method, described below. A further technique of validation used was microscopical examination; actually seeing the lymphoblasts was comforting.

Separated Lymphocyte Technique

Blood anticoagulated with Heparin was separated to yield a predominantly lymphocyte population using Ficoll hypaque and centrifugation. The lymphocytes were washed three times in PBS and resuspended in Medium 199 with 10% autologous serum at a concentration of $1x10^6$ cells/ml. Incubation at 37^oC for 48 hours in CO_2 5% and air was followed by a period of 8 hours labelling results being expressed as DPM.

The Effect of Granulocytes

Buffy coat cells were separated on a Ficoll hypaque gradient, the

granulocytes were washed, and resuspended in Medium 199. They were then added to the lymphocytes (at a ratio of 1:1) or boiled for 10 minutes before being added. Control and PHA stimulated values are shown in the Table. The addition of either live, or dead granulocytes improved the PHA response. Whether the granulocytes were dead or not was statistically indistinguishable.

Microscopic Appearances

Lymphoblast appearance following PHA stimulation could always be observed in either whole blood or lymphocyte cultures, provided that the culture period was sufficiently protracted. No lymphoblasts occurred in control cultures.

Amino Acid Protein Incorporation

Method:

Blood was taken from 2 subjects known to respond to PHA and lymphocytes obtained. Following dilution and culture, aminoisobutyric (C14) or Leucine (C14) was added at 1, 6, 12, 24 and 48 hours for labelling periods of 4, 12 or 24 hours.

Only at 24 hours and 48 hours with 24 hour labelling was a significant difference between control and stimulated cultures observed. There was close correlation between the DPM and even the significance achieved with either AIB Leucine or thymidine incorporation at the times 24 and 48 hours.

Separated B and T Cells

Many workers have reported techniques to separate B from T cells. T cell innumeration is simple and the techniques can be checked by

Table 12 Effect of Addition of Granulocytes

Granulocytes	Control DPM Mean ± ISE	PHA DPM Mean ± ISE
None	717 ± 120	66,113 ± 6,951
Dead	899 ± 113	95,121 ± 9,800
Live	875 ± 107	93,776 ± 8,678

$p < 0.01$ compared with none

TABLE 13 Effect of PHA on $AIBC^{14}$ Incorporation

Time of Addition	Time of Incubation	Control DPM Mean ± ISE	PHA DPM Mean ± ISE
1	4	54 ± 7	60 ± 11
	12	603 ± 58	571 ± 61
	24	584 ± 53	594 ± 68
6	4	61 ± 9	63 ± 13
	12	664 ± 73	660 ± 79
	24	659 ± 77	594 ± 88
12	4	73 ± 16	95 ± 21
	12	576 ± 45	631 ± 65
	24	613 ± 81	599 ± 77
24	4	114 ± 22	110 ± 24
	12	770 ± 53	1495 ± 120*
	24	721 ± 49	1666 ± 100*
48	4	129 ± 81	140 ± 52
	12	897 ± 49	1910 ± 202*
	24	860 ± 45	2415 ± 212*

TABLE 14 Effect of PHA on Incorporation of C^{14} Leucine

Time of Addition (h)	Time of Incubation (h)	Control Mean ± ISE	PHA Mean ± ISE
1	4	132 ± 26	126 ± 22
	12	162 ± 26	187 ± 21
	24	190 ± 21	201 ± 31
6	4	136 ± 5	127 ± 31
	12	106 ± 17	141 ± 40
	24	211 ± 21	199 ± 25
12	4	141 ± 12	130 ± 38
	12	142 ± 29	88 ± 41
	24	249 ± 61	211 ± 71
24	4	166 ± 9	143 ± 21
	12	121 ± 16	133 ± 20
	24	247 ± 18	599 ± 91*
48	4	149 ± 17	148 ± 36
	12	155 ± 13	515 ± 62*
	24	230 ± 20	712 ± 66*

* p 0.01 compared to the control

alterations in T cell counts. The production of normally responding cells, however, is more difficult. Most separation techniques must produce stimulation since they utilise Ficoll-hypaque gradients, foreign red cells (sheep) and pooled human serum. Small differences in stimulation are probably masked by these means.

Methods

1. Production of T cell depleted lymphocytes

Whole blood, heparinised, was layered on Ficoll-hypaque, centrifuged, the lymphocytes aspirated and washed. T cell rosettes were formed by culture at $4^{o}C$ overnight with fresh sheep red cells. The following morning the cultures were layered onto Ficoll-hypaque, centrifuged and the rosette deplete lymphocytes aspirated and washed three times.

Further rosette tests on these lymphocytes revealed a 10% ability to form rosettes.

2. Production of B cell deplete lymphocytes

Ficoll-hypaque separated lymphocytes were incubated for one hour in a pipette filled with nylon fibre coated with AB serum following which Medium (TC199) was infused through the pipette, and the resulting released lymphocytes, predominantly T cells, collected.

About 90% of these cells produced rosettes. Predominantly T, and B lymphocyte cultures.

Following the preparation of the T or B cell deplete population, the cells were washed and suspended at $1x10^{6}$ in culture medium. Following 24 hour culture thymidine was added for 4 hours.

Initially repeated cultures showed inordinately and unacceptably high control counts. I could not determine if they were due to bacterial contamination, which with so many steps is possible, or to stimulation occurring during the separation procedure. Bacterial culture was negative.

I have repeated these experiments having left the lymphocyte mixture for 24 hours at $4^{o}C$ and then washed x3. This procedure improved the method - possibly by allowing ligands or ligand effects to be removed.

Results

Predominantly T cell.

	Mean	SEM
Control	11,550	2,659
PHA	91,566	10,144 (p 0.01)
Dextran Sulphate (MWt. 500,000)	14,113	3,175 (p 0.05)

5 experiments; separated lymphocytes; preponderantly T cell; Means ISEM.

Predominantly B cell.

	Mean	SEM
Control	14,156	3,004
PHA	13,987	2,967
Dextran Sulphate (MWt. 500,000)	21,500	5,999 (p 0.01)

6 experiments; separated predominantly B cell lymphocytes; Means ISEM.

The tables show that PHA induced a significant increase in counts only in the 'T cell' preparation. Dextran Sulphate (a known B cell mitogen, Diamanstein et al 1973) produces slight increases in T cell population, but much more in the 'B cell' population.

Conclusion

These results suggest reasonably pure populations of lymphocytes; the raised control values still imply activation during the separation, which must decrease the value of the method.

FIBRINOLYSIS

Hunter observed that the blood of chased deer failed to clot, confirming the observations of Morgani (1976) and Davy & de Senai (1749) that blood is fluid and incoagulable after sudden death; the rationale for a corpse blood transfusion (Judine 1936). These observations were responsible for the idea that fibrinolysis was unusual. The views of Paul Nolf (1900) were ignored; he had postulated a dynamic equilibrium between fibrin deposition and removal by a labile fibrinolytic agent.

The discovery of streptococcal fibrinolysis (Tillett & Garner 1933) with characterisation of the resultant fibrinolytic system by Christensen & Macleod (1945) directed thought away from spontaneous fibrinolysis to that enhanced by streptokinase. The plasmin scheme was evolved.

Streptokinase combines on an equimolar basis with plasminogen to produce the stoichiometric complex plasminogen activator. This protease is fibrinolytically inactive but activates plasminogen to plasmin. (see figure)

Activation of Plasminogen

Activator	Plasmin
Glu-plasminogen $\xrightarrow{\text{Activator}}$ Glu-plasmin (1)	Glu-plasminogen $\xrightarrow{\text{Plasmin}}$ Lys-plasminogen (3)
Lys-plasminogen $\xrightarrow{\text{Activator}}$ Lys-plasmin (2)	Glu-plasmin $\xrightarrow{\text{Plasmin}}$ Lys-plasmin (4)

To account for the rapidly damped system the complex plasmin inhibitor system was suggested and identified (Ratnoff et al 1954; Norman 1958). To explain how plasmin could exist in a potentially

hostile environment, Sherry et al (1959) postulated that plasminogen was absorbed onto fibrin with a competitive affinity for plasmin between fibrin and plasmin inhibitors. Ambrus & Markus (1960) suggested that fibrinolytic activation occurs predominantly in the circulation, plasmin being immediately bound to inhibitor but this resulting complex dissociates on coming into contact with fibrin, liberating free plasmin. The most attractive hypothesis (Chesterman et al 1972) is that activator binds to fibrin; plasminogen perfusing the thrombus is then activated locally; transformation of fibrinogen to fibrin is responsible for the adsorption of plasmin (Aoki N, 1979)

While these American workers characterised streptokinase fibrinolysis, elsewhere inquiry pursued more physiological lines. Biggs et al (1947) showed that injections of adrenaline or violent exercise produced activity in dilute plasma and soon fibrinolysis was demonstrated in normal blood (Fearnley & Tweed 1953). The concept that spontaneous fibrinolysis was mediated via plasmin activation was not entirely satisfactory because in Fearnley's experiments (Fearnley et al 1952; Fearnley & Tweed 1953; Lackner 1955) the responsible fibrinolytic activity called 'labile fibrinolytic component' was stabilised by clotting, inhibited by calcium ions, and heat labile; unlike plasmin. Sawyer et al (1960) showed that fibrinolysis correlated with the amount of contaminating plasminogen and was inhibited by EACA; features thought to be consistent with 'plasminogen activator'. Five fibrinolytic mechanisms have been delineated in plasma. The classical pathway relies upon Hageman Factor activation, with activation of plasminogen preactivator resulting in plasmin generation. A Hageman Factor independent pathway has been described, involving C3 but possibly no plasmin (Schreiber & Austen 1974); another pathway relies on participation of platelets,

Igm, C3 and C4 and possibly plasminogen (Taylor & Muller Eberhard 1970). Polymorphonuclear leucocyte proteases (Plow EF & Edgington TS 1975) are fibrinolytic but do not utilise plasminogen; the fifth is vasular activator

Moroz & Gilmore in 1976 showed that spontaneous basal fibrinolysis was not greatly affected by removal of plasminogen or plasmin or its inhibition. It could be significantly reduced by agents or manoeuvres which do not alter plasmin such as heat inactivation or zymosan treatment. There was no effect due to glass contact, suggesting a minor role for Hageman Factor dependent fibrinolysis. They proposed that spontaneous fibrinolysis is mainly due to complement activation but also involves complement control proteases such as C3b inactivator. This normal plasma protein controls the level of C3b by proteolysis to C3c and C3d (Ruddy, Austen 1969). This historical approach has perhaps under emphasised the importance of assays for fibrinolysis. Although of limited clinical importance each new conceptual advance has been necessitated by observations culled from improved methodology.

Until the description by Moroz & Gilmore, available methods of investigation were all limited by insensitivity, by being excessively time consuming and unphysiological. The initial problem was partly that of recognition; if blood is allowed to clot in a test tube and incubated at 37°C, the clot will remain solid for days or weeks. Dilution of blood or plasma hastens fibrinolysis, possibly because of the diminished effect of inhibition (Macfarlane 1937), or because of alteration of the quality of the clot. The lysis time is also affected by the clotting agent since clots of dilute blood or plasma with thrombin in the absence of calcium (preventing cross linking of

fibrin) reliquify within a few hours of incubation, whereas if calcium is present the lysis time is 2-3 times longer (Fearnley & Tweed 1953; Lackner 1955; Ferguson 1958).

Fearnley (1961) has said that the measurement of the dilute whole blood, or euglobin clot lysis time is crude. Inherent in this method is an acidification artefact altering the effectiveness of inhibitors and proteases. The other available methods; namely lysis of radioactively or fluorochrome labelled fibrin clots (Alkjaersig et al 1952; Sawyer et al 1960; Genton et al 1964) or of fibrin gels in the fibrin plate method (Lassen 1952; Astrup, Mullertz 1952) are insufficiently sensitive to detect modulation of the function of plasmin either directly or by influencing proenzyme activation; fail to monitor activators or inhibitors which enter the substrate poorly; and do not provide adequate separation of reaction products from residual substrate.

The technique of Moroz & Gilmore fulfil most criteria for an assay of fibrinolysis; I have modified this technique slightly and separately validated this modification. Simply, the method relies on the absorption of I^{125} labelled fibrinogen onto polystyrene tubes, which is then clotted. Blood or plasma is added to the tubes for known time, and an aliquot of this is then counted in a counter. The results can be expressed as ng fibrin lysed/ml/hour. Moroz & Gilmore found that it reproduced certain established features of plasmin function (effect of inhibitors, streptokinase and urokinase activation). It was technically convenient, assays may be completed within 30 minutes (a marked improvement on the 6 hour minimum required by the fibrin plate), the method was highly

sensitive (x10 greater than the fibrin plate method) with low background levels (15.2ng±1.2 SE) (I^{125} fibrin lysed/ml/hour) and permitted a sensitivity of 30-50 ng fibrin.

There are potential difficulties with this assay since the firbin is not cross linked as the clotting procedure is performed in the absence of calcium. This I believe is advantageous because it increases the liklihood of fibrinolysis and hence the sensitivity of the procedure. Experiments performed by Moroz & Gilmore and duplicated by myself have shown that venous blood added to the fibrin tubes and allowed to clot produces similar results to EDTA treated blood. Since this non-anticoagulated blood contains excess calcium, conversion of the labelled fibrin to cross linked may occur; suggesting that cross linking is not of major importance to the results. The method as described by Moroz & Gilmore does not allow the substrate concentration to be varied. This was no problem in a clinical situation, it simply meant that kinetic analysis requiring substrate concentration variations were impossible.

Methodology

I shall describe the method I adopted and outline the areas where my method varies from that of Moroz & Gilmore.

Preparation of I^{125} Fibrin coated tubes

Disposable polystyrene tubes (LP3) were used. For coating the tubes I^{125} fibrinogen (human, Amersham) solution (I) was prepared into a 0.015M phosphate buffer of ionic strength approximating 0.1M. Into each tube was pipetted 0.1ml of the fibrinogen solution representing 10 ug of I^{125} fibrinogen. The tubes were rotated in a tissue culture

rotator at room temperature and an angle of 45° for 3 hours. The unbound fibrinogen was then aspirated (2) and the tubes washed four times with 4ml aliquots of Tris NaCl (0.015M Tris, 0.15NaCl pH 7.4) buffer.

After aspiration to near dryness, 0.2mls of Tris NaCl buffer containing 2U of bovine thrombin (3) (substantially free of plasminogen contamination) were pipetted into each tube, and the tubes incubated at 37°C for 15 minutes. After aspiration of the thrombin solution tubes were washed x2 with Tris NaCl buffer, filled with buffer and stored at 4°C until required, when they were again washed with Tris NaCl.

Blood Samples

Venous blood was collected with the use of a tourniquet applied for a maximum of 1 minute, vacuum tubes in common use (Vacutainer, EDTA anticoagulant), and immediately placed on ice.

Fibrinolytic Assay

Assays were performed in quintuplicate; appropriate buffer controls being performed and counts subtracted from experimental values throughout. 200uls of blood and an equal volume of Tris NaCl buffer were added to each tube. The tubes were incubated at 37°C in an atmosphere of 5% CO_2 for 30 minutes, following which the reaction was terminated by rapid vigorous addition to each tube of two 200ul aliquots of cold Tris NaCl buffer. The tubes were then vigorously shaken, a 200ul aliquot removed to a new uncoated LP3 tube and counted in a gamma scintillation spectrometer. The results were expressed as ng fibrin lysed/ml/hour, mean $\pm$ ISE.

Differences in Methodology

There were three variations:-

(1) In the technique of Moroz & Gilmore the human fibrinogen was freed of contaminating plasminogen by passage through a lysine-Sepharose column. This I thought was unnecessary in a clinical test, but experiments to validate this conclusion were performed.

(2) Moroz & Gilmore used Bovine serum albumin to bind any polystyrene sites unoccupied by the I^{125} fibrinogen. Omitting this stage increased the possibility of I^{125} fibrin breakdown products absorbing onto the unoccupied sites. This seemed theoretically unlikely since the proteins present in the test whole blood would be also absorbed onto the polystyrene sites; since the concentration of these were greater than that of the fibrin breakdown products any effect would be minimal.

(3) Bovine thrombin was used which was commercially prepared and substantially free from plasminogen contamination. Moroz & Gilmore purified impure Bovine thrombin.

Validation of Changes in Method

Plasminogen contamination.

If significant contamination of the fibrin monomer with plasminogen occurred, then incubation with streptokinase would produce fibrinolysis. Experiments were performed on three separate batches of 8 tubes; serial dilutions of streptokinase (courtesy of I.C.I. Limited) from 0.1 units/ml to 1000 units/ml were added in buffer to the tubes which were incubated for the 30 minutes. There was no significant difference from control tubes containing buffer alone, despite whole blood or serum showin a 10 to a 100 fold

increase in fibrinolysis.

TABLE Experiments to Determine Plasminogen Contamination

	cpm of fibrin I^{125} lysed/200ul		
	Mean c.p.m.	±	ISE
Tris NaCl	97	±	9
-Streptokinase 0.1Iu/ml	98	±	10
10 u/ml	96	±	8
100 u/ml	95	±	9
1000 u/ml	100	±	11

This demonstrated that any plasminogen contamination was insignificant and also justified the use of the commercially purified thrombin.

Plasminogen activator contamination.

Just as plasminogen could contaminate the fibrin, so could plasminogen activator. I therefore performed experiments using commercially obtained procine plasminogen (Sigma). The results in the table show that at very high concentrations of plasminogen there was a significant increase in fibrinolysis as expressed as cpm of fibrin I^{125} lysed/200 uls. These concentrations were about 100 times greater than the minimum amount of plasmin needed to enhance fibrinolysis and are also compatible with plasmin or plasminogen activator contamination of the Sigma plasminogen.

TABLE Effects of Porcine Plasminogen

Means of 4 experiments on separate batches of tubes.

	cpm mean	±	ISE
Tris NaCl	93	±	6
-plasminogen 1 ug/ml	91	±	7
10 ug/ml	92	±	8
100 ug/ml	101	±	11
1000 ug/ml	141	±	19

significance, Mann Whitney 'U' test $p < 0.05$, compared with control.

Bovine serum albumen.

It would be odd if the small number of fibrin degradation product (FDP) molecules produced during the assay would significantly bind to the unoccupied binding sites on the polystyrene tubes because the time exposed is short and the FDP quantity would be vastly smaller than that of other proteins in blood likely to bind to polystyrene. To confirm this a priori reasoning an experiment was performed comparing tubes made with exposure to bovine serum albumen (BSA) and made as previously described. The fibrinolytic activity of 10 subjects and the coefficient of variation was determined and is shown in the table below.

Method	Mean Fibrinolytic Activity	Coefficient of Variation
with BSA	242 ng/ml/hour	9.8%
modified	249 ng/ml/hour	8.8%

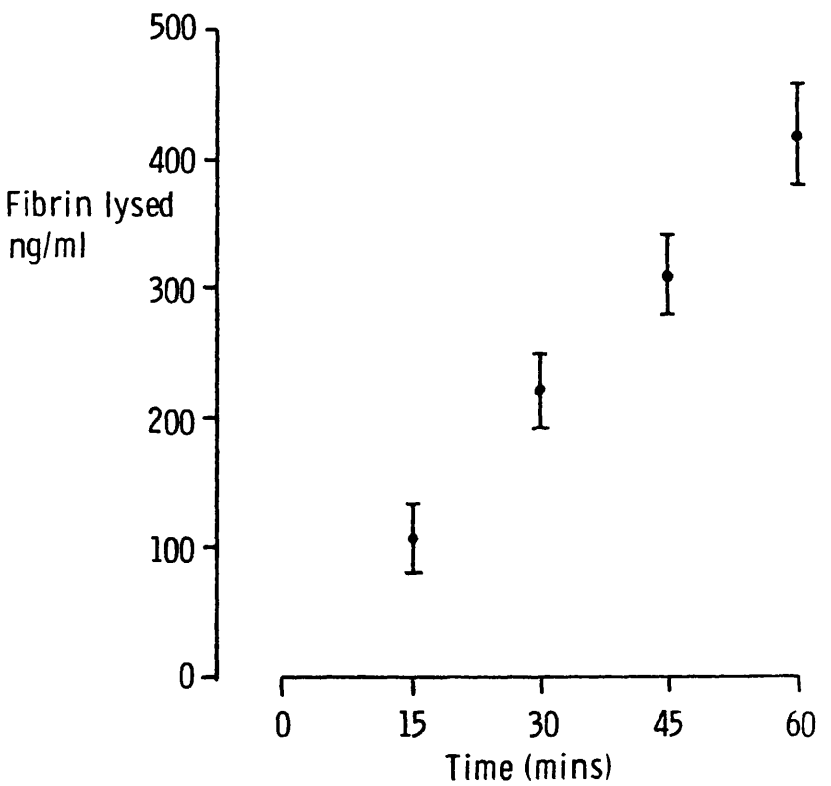


Fig: Linear Generation of Fibrin Degradation Products (mean ± 1SE)

Properties of the I^{125} fibrinogen to the polystyrene tubes was reproducible, both within (coefficient of variation ± 7.5%) and between batches (coefficient of variation ± 8%). Following treatment of the absorbed fibrinogen with thrombin there was a release of about 5.7% of the bound fibrinogen, consistent with the release of fibrinopeptides A and B. The vigorous washing with Tris NaCl buffer released small (0.8%) of the bound radioactivity. These results were similar to those achieved by Moroz & Gilmore.

Nonspecific Release of Fibrin

After 30 minutes incubation of tubes with Tris NaCl buffer there was never greater than 20ng I^{125} fibrin lysed/ml/hour and over 20 experiments the mean was 16.2 ± 1.6 ±ISD)ng fibrin lysed/ml/hour. This would suggest insignificant plasmin contamination, and represents the limits of sensitivity of the assay.

The Effect of Time of Assay

Whole blood from 3 volunteers was assayed at various times as in the Figure. This confirmed the findings of Moroz & Gilmore that the assay was linear over an hour. Readings at 30 mins were found to be the most convenient.

Substrate Dependence

Five experiments on whole blood from volunteers were performed, 20 replicates were incubated for 30 minutes and the amount of fibrin lysed determined. Prior to the assay, however, the fibrinogen content of each tube was determined by γ counting. Regression of fibrinogen to fibrin lysed was, however, not significant (R - 0.145) nor was it significant in any individual experiment. It would appear therefore,

that the reaction is substrate independent under these experimental conditions.

The Effect of pH

The enzyme systems involved in fibrinolysis are significantly affected by changes in pH. In the method used blood is buffered with Tris NaCl, but obviously it is theoretically interesting to examine the effect of pH change in fibrinolysis, and practically important to ensure that blood in acidotic patients is adequately buffered. (In investigations upon acidotic individuals it would also be important to interpret the effect of the acidosis upon fibrinolysis.)

The Degree of Buffering Achieved in the Assay

Venous blood from 6 volunteers was each divided into several aliquots. In one, fibrinolysis assay and pH measurement were performed immediately following dilution with Tris NaCl buffer. To other aliquots of the blood, either dilute hydrochloric acid with mixing (if haemolysis occurred the experiment was abandoned) or dilute sodium hydroxide was added to ensure a pH range from 7 - 7.8. 200 uls of this blood was then added to 200 uls of Tris NaCl buffer, the pH measured and fibrinolysis assayed. Despite these steps the pH of the buffer-blood mixture remained constant, and there was no significant effect upon fibrinolysis.

The Effect of pH Changes on Fibrinolysis

A similar method was adopted, using blood from 4 volunteers but instead of diluting the blood with Tris NaCl buffer, it was diluted

with isotonic saline. As the pH fell to 7.0 there was a marked and significant ($p < 0.001$) increase in fibrinolysis, and there was a slight but significant ($p < 0.05$) fall in fibrinolysis with the pH rise to 7.8.

This would be expected simply from the known optimal pH of plasmin which is about 7.0. Other determinants at lower pH were made. These were not strictly needed for the validation of the technique, but were interesting. Peak fibrinolysis occurred at a pH of 5.8, with a range of 5.5 - 6.4. This must be further evidence against plasmin as the only possible fibrinolytic enzyme.

These findings were confirmed in an analysis of 4 patients with diabetes who were admitted in diabetic ketacidotic coma. Fibrinolysis was reduced until corrected for the intense acidosis, when it was slightly raised.

Storage of Blood

Obviously some storage of blood would be useful before assay. In common with most methods the blood was put into an ice bucket immediately. Fibrinolysis was measured in 6 subjects, either immediately after the blood was taken, or at hourly intervals for 4 hours. The results are shown in the Table where it can be seen that storage has little effect on the result.

TABLE

Time	Fibrinolysis ng/ml/hour Mean ± ISE
0	256 ± 12.5
1hr	261 ± 18.9
2hr	251 ± 15.1
3hr	263 ± 22.9
4hr	266 ± 12.8

Standardisation

Two techniques were used to ensure adequate standardisation. Plasmin standard curves were produced, and, as can be seen in the figure, on 4 separate batches of tubes there was close agreement. The amount of plasmin detectable, 0.2 ug/ml is considerably less than in other conventional techniques (2ug/ml).

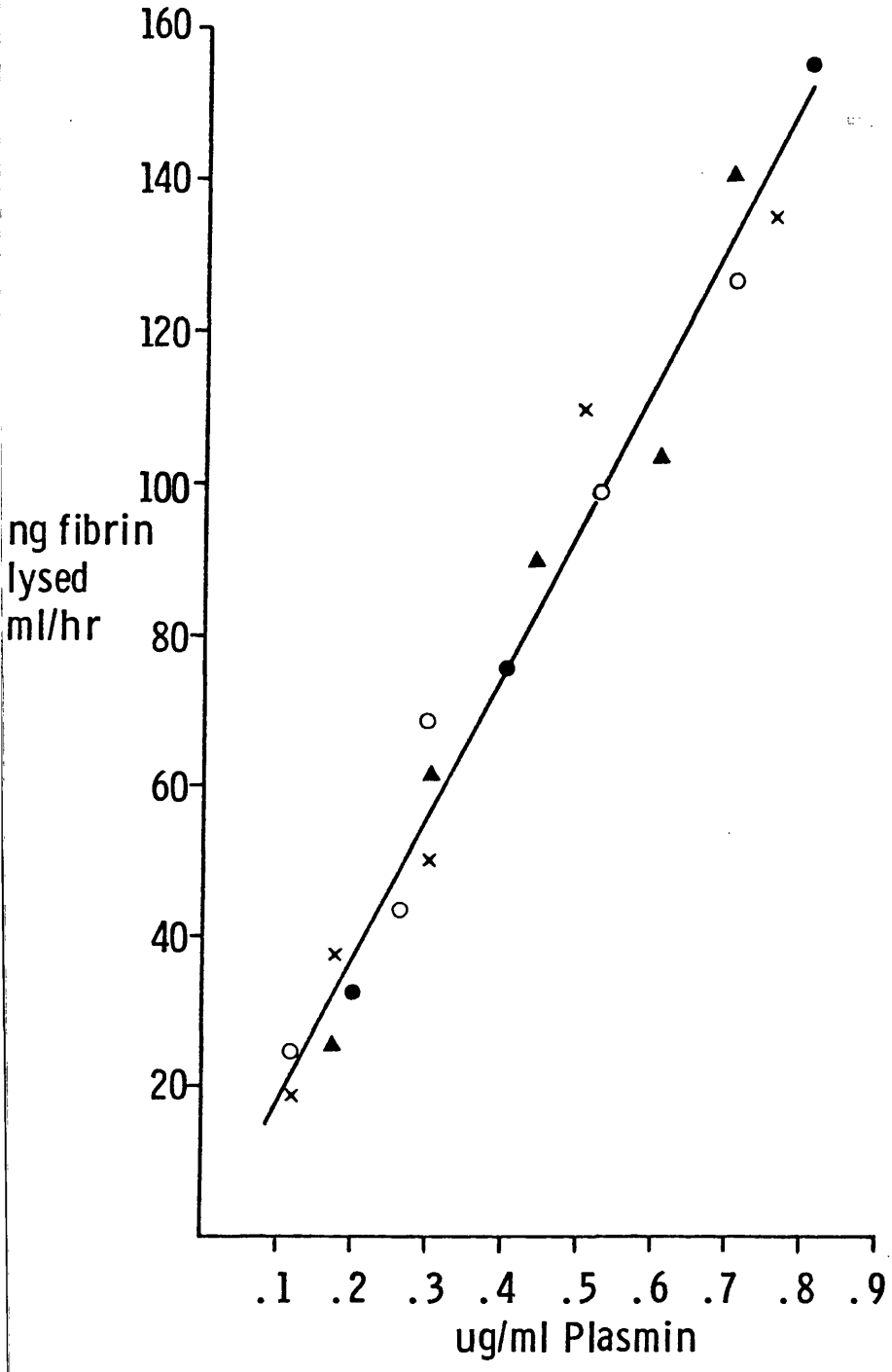
Pooled human serum divided into 1 ml batches and stored at -23°C was also used to ensure adequate standardisation. The co-efficient of variation as assessed using this serum was 8.5% over 10 batches of tubes.

Summary

The results show adequate validation of the modifications. The assay remains simple and accurate especially in comparison with other techniques for measuring fibrinolysis.

Fibrinolysis in Systems Other Than Whole Blood

It was obviously useful to measure the effects of streptokinase or protease inhibitors upon the tests of cellular immunity. This was



Standard Curve by the I^{125} fibrin tube method: results of 4 seperate experiments, one standard curve for each of 4 different batches of tubes. Each point assayed in quadruplicate.

convenient in the method used to examine lymphocyte transformation since this used a dilute whole blood technique, but the LMT used 10% foetal calf serum in medium, and the animal tumour studies were performed in mice. Although not ideal it seemed reasonable to use the present test in both situations.

Fibrinolysis in LMT

(1) In an experiment to find the validity of the I^{125} fibrin test in this situation, the amount of fibrin lysed was studied at time intervals from 0 to 60 minutes in medium containing 10% foetal calf serum, and in such a medium containing polymorphonuclear leucocytes. The results shown graphically in the Figure reveal linear lysis with a minor variation between control assays. At each time analysis, there was significantly increased fibrin breakdown in the medium containing PMNL.

(2) The addition of streptokinase to this system significantly enhanced fibrinolysis, but only when at much higher concentrations than in whole human blood.

Summary

The fibrinolytic assay can therefore be used in the LMT. The finding that the dose response curve to streptokinase is shifted to the right in the LMT compared with human whole blood simply supports the known insensitivity of calf serum to the drug.

Mouse

(1) Mouse whole blood showed a linear response in lysis against time up to one hour. There was only a small variation, and mice of the same age, sex, weight and habit had comparable fibrinolytic activities.

(2) Streptokinase. - There was a highly significant increase in fibrinolysis with the addition of streptokinase, maximal at a concentration of 125 iu/ml (456 ± 26 fibrin lysed/ml/hour before S.K. 1289 ± 119 lysed/ml/hour with streptokinase).

Summary

Mouse whole blood is capable of producing fibrinolysis of human fibrin. This was appropriately amplified by streptokinase added in vitro. This latter finding has not been previously reported.

Conclusion

The I^{125} fibrinolytic assay was found to be an exact measure of human fibrinolysis. It could be used to measure fibrinolysis in the LMT, and in mouse blood.

Fibrinolysis

Before the fibrinolytic activity of patients undergoing surgery could be assessed it was felt necessary to define the conditions affecting fibrinolysis, and to define the distribution of fibrinolysis in the general population.

Several factors are known to affect other tests of fibrinolysis. The most careful work in the general population is probably that of the prospective Northwick Park Heart Study (Meade et al 1977) where subjects were recruited from the main London production site of a food processing company. Among the variables examined was fibrinolysis, using the dilute blood clot lysis time of Fearnley & Chakrabarti. The lysis times were not normally distributed (the skew

to the left being partly due to artificial constraints imposed by the method). There was a small laboratory variance (8% of total variance) a larger inter persons variability (19%) but an enormous whithin person variability (73%) which would hinder any analysis. Since about 2000 people were examined it was still possible to conclude that age affected fibrinolytic activity in the male, with old people having less activity; higher social classes had lower activity; cigarette smokers had lower activity (males only) and women on the contraceptive pill had a higher fibrinolytic activity.

Other studies had also demonstrated that various factors could affect fibrinolysis; strenuous exercise (Davis et al 1976); drugs such as adrenaline [illegible] or biguanides (Fearnley & Chakrabarti); the time of day (Fearnley)[a]; or if the patient was diabetic (Almer et al 1975) or had peripheral vascular disease.

It seemed reasonable to examine these factors to see if the mentioned variation in fibrinolysis could be reduced by standardising the test.

The Effect of Exercise

Blood was taken from 5 volunteers after 30 minutes rest before exercise, after a 15 minute brisk walk on a treadmill, and after a 15 minute run on a treadmill at 10°.

Fibrinolytic activity was 161 ± 22 (ISEM) ng/ml/h at rest, 165 ± 32 (ISEM) ng/ml/h after walking; an insignificant difference an; 262 ± 41 ng/ml/h after running; significant ($p < 0.05$ paired t test).

For the fibrin matrix test walking had little effect in fibrinolysis; ambulatory fibrinolytic activity was therefore satisfactory.

Diurual Variation

Blood was taken from 15 volunteers at 9am, 12md, 2pm and 4pm. In 6 individuals there was a significant change in fibrinolysis (judged by the Mann Whitney U Test on the quintuplicates); this variation did not fit with any direct dirual pattern but simply reflected times of severe stress (operating : 3 subjects; cardiac arrest calls : 2 subjects; phone call relating to death of her cat : 1 subject). It is known that operating sessions (at least) produce adrenaline release, adrenaline increases fibrinolysis (Tanser & Smithe).

To assess the effect of sleep blood was taken from 4 patients - who had given their consent - immediately before the night time sedation round, at 12 midnight after the patients had been asleep for 2 hours; at 6am and 9am.

Fibrinolytic activity was significantly lower at 12 midnight and 6am than at the other two times - see Table.

TABLE Effect of Sleep on Fibrinolysis

	Fibrinolytic Activity ng/ml/hr Means ± ISEM
10pm	247 ± 43
12mn	151 ± 32
6am	144 ± 38
9am	261 ± 48

TABLE Effect of B Blockade on Fibrinolysis

Treatment	Basal Pulse Rate During Treatment	Fibrinolytic Activity	
		Before Treatment	After Treatment
1. Propranolol 160mgs	64	253	121
2. Oxprenolol 160mgs	72	198	176
3. Propranolol 320mgs	42	173	98
4. Propranolol 80mgs	54	72	51
5. Atenolol 100mgs	62	232	215
6. Oxprenolol 160mgs	68	187	188
7. Propranolol 480mgs	48	215	211
8. Propranolol 320mgs	52	267	98

Drugs

It was relatively easy to confirm the effects of drugs known to markedly affect fibrinolysis; Salbutamol, a B_2 agent, was given to 5 volunteers. Blood was taken before and 2 hours after a 4mgm oral dose. Fibrinolytic activity before Salbutamol was 155 ± 41 (ISEM) ng/ml/hr but afterwards was 461 ± 89 (ISEM) ng/ml/hr, a highly significant ($p < 0.001$) effect.

Phenformin is now no longer used by me (because of the problem of lactic acidosis) but in 10 obese diabetics, only reasonably controlled with dietary restrictions alone, Metformin (mean dose 120mg daily, range 500-160mg) produced a meagre increase in fibrinolytic activity. Before treatment it was 79 ± 22 (ISEM) ng/ml/hr but two weeks after had increased to 116 ± 39 (ISEM) ng/ml/hr, $p < 0.05$ 'U' test.

Thiazide diuretics appeared to have no effect, but B blockade, had a significant effect ($p < 0.05$) decreasing fibrinolysis. (see previous table)

I was not able to examine the individual effects of anaesthetic agents upon in vivo fibrinolysis, but of the other assaults that the body copes with during surgery, blood transfusion must rank quite highly. Several patients who were undergoing transfusions for anaemias of 'medical' origin were studied. Fibrinolysis was determined before, during and after transfusion. There was an increase in fibrinolysis during the transfusion which decreased subsequently as can be seen in the Figure.

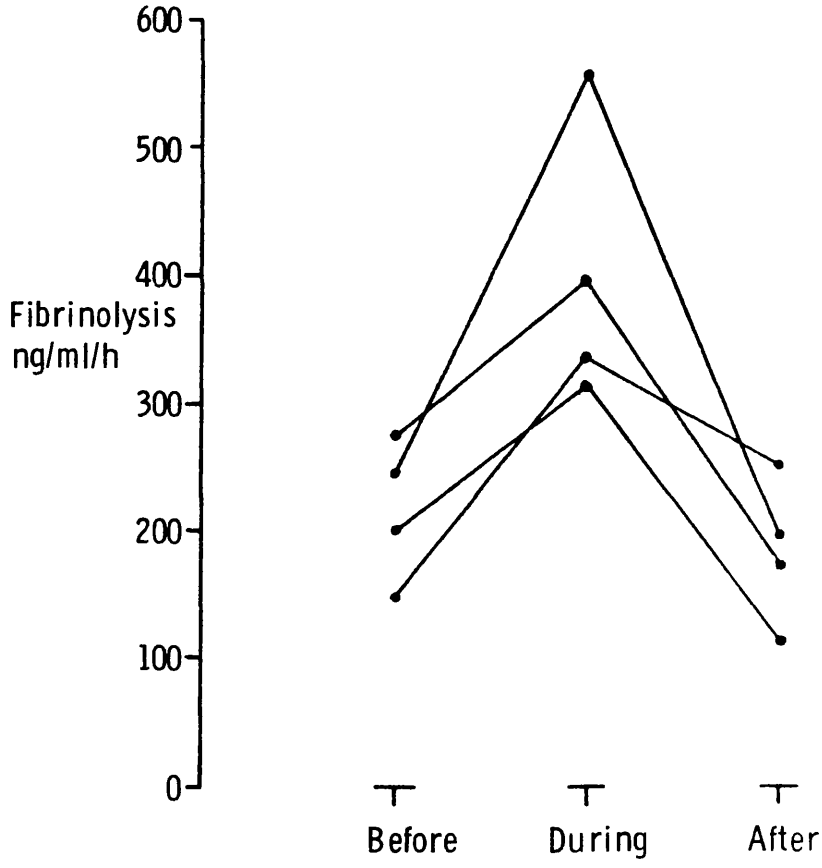
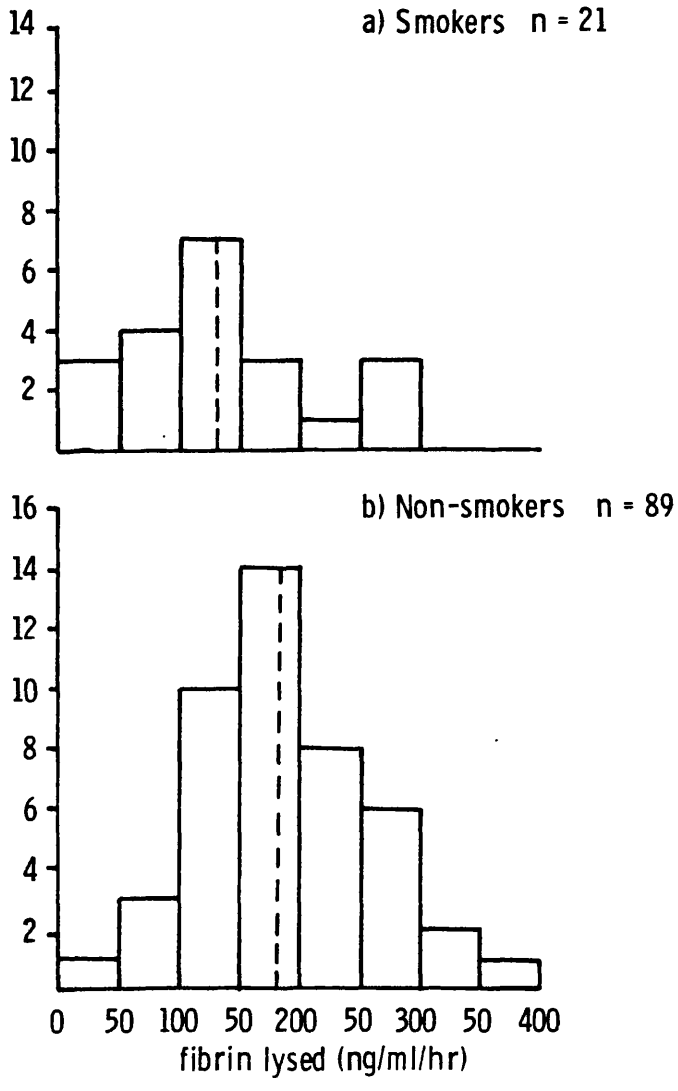


Fig: The Effect of Blood Transfusion

FREQUENCY DISTRIBUTION OF FIBRINOLYTIC ACTIVITY IN CONTROL POPULATION DIVIDED INTO SMOKERS AND NON-SMOKERS



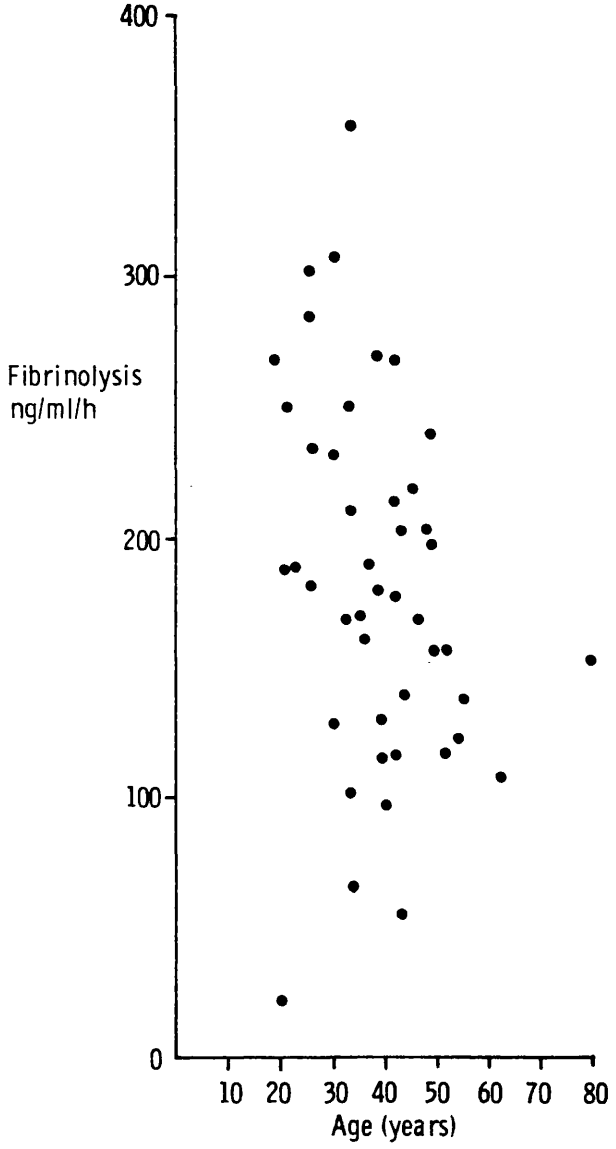


Fig: Age and Fibrinolysis
R 0.34, n = 45, $p < 0.05$

Normal Population

To examine fibrinolysis in the normal population, members of the hospital staff, and patients attending a surgical outpatients with complaints unlikely to affect fibrinolysis (irritable colon syndrome, herniae) were asked if they would allow a venepuncture for this study. If they agreed venous blood was obtained as previously described. They were also asked about their consumption of cigarettes, alcohol and drugs. From this total population was selected those people who did not smoke; took no (admitted) drugs; and drank no more than one pint (or equivalent) a day. These formed the control population (see figure) which showed normal distribution. Smokers had a significantly ($p < 0.05$) lower fibrinolytic activity 134 ± 84.6 (ISD) ng/ml/hr than the non-smoker group 182±71 (ISD) ng/ml/hr. The drinkers, not all doctors, had a raised fibrinolytic activity, 234±72 (ISD) ng/ml/hr ($p < 0.05$). Those taking the oral contraceptive pill had a slight increase in fibrinolysis; comparing smoking women oral contraceptive takers with non-oral contraceptive takers, and non-smoking oral contraceptive takers with the relevant control showed a significant effect ($p < 0.05$ for each group).

Age produced a significant decrease in fibrinolysis (see figure) R-0.34 n 45 $p < 0.05$.

Conclusion

The normal population; ambulatory; and taking no drugs was defined. For comparison with other subjects the effect of smoking, drinking and several commonly taken drugs was found.

THE ANIMAL MODEL

This is not intended as a review of techniques developed to explore oncology in the animal model. The subject matter is too great; I would rather analyse the Lewis Lung Model.

This tumour originated as a sarcoma preferentially metastasizing to the lung, it occurred in the Black C57. It appealed as a useful model because of the short natural history. Unlike some carcinogenically induced tumours the results in previous workers hands showed that the technique was reproducible.

The main criticism are those of any animal model: the species difference compared with humans; and the frequent poor statistical handling of results. Those criticisms specific to this model hinge upon the reputedly low immunogeneity and that the frequency of lung metastases do not necessarily correlate with the frequency of body metastases.

I have attempted to answer some of these criticisms.

Method

Ether anaesthetised C57 Black Mice which were three weeks old were innoculated with 0.1 ml of tumour subcutaneously into the flank. About 10 days later the tumour was resected under ether anaesthesia. At 4 weeks the animals were killed, a thoracotomy performed, the lungs filled with carbon (Pelican Ink), removed, and fixed in Fekhetes Solution.

Tumour passage was performed by leaving the animals for 2-3 weeks

after tumour innoculation before removing the tumour. Infected growths were destroyed, necrotic areas discarded, and the technique performed under sterile conditions - which lessened infection. The tumours were finely minced using scissors, 0.1 ml of antibiotic (penicillin and streptomycin) added and the tumour drawn into a 1 ml syringe. By repeated aspiration the tumour was rendered fine enough to be injected through a serum needle. About three tumours were used for each sub-group; to amalgamate more increased the risk of infection.

Variables

1. Site of Injection

Flank S.C. injections occasionally resulted in peritoneal extension and ascites, with subsequent rapid death. A loin site was attempted, but produced a more variable response in lung metastases.

2. Volume of Tumour

	Size of Tumour at 2 weeks (Mean)	Necrosis
0.05 mls	2 x 3 cms	+
0.1 mls	2.5 x 3 cms	+
0.2 mls	2 x 3 cms	++

Three volumes were used, and although the smallest quantity gave equal sized tumours at 2 weeks with minimal necrosis, there was a high non-acceptance rate; 0.1 ml volume was chosen.

3. Antibiotics

An old fashioned regime of penicillin and streptomycin was used.

This was effective in reducing the number of infected lesions; and did not decrease either tumour primary volume or frequency of metastases.

4. Tumour Resection

Initially I tried not removing the tumour, but performing a mock laparotomy. This certainly enhanced primary growth and number of metastases when compared with ether anaesthesia alone; which itself increased the number of metastases. One major problem was that the mice tended to die earlier than the planned three weeks.

The more exact technique was resection of the small C10 day tumour which whilst it had an operative mortality, this was small (4%) and mainly represented anaesthetic problems.

Primary Recurrence Rate

The largest determinant was the pre-resection size of tumour.

Variability of Metastases

One problem with this model is the variability of metastases which is inherent with the small tumour pooling needed to reduce the risk of infection. This occurs with the work of others (for example, Castro et al) who confuse matters further by using parametric statistical tests when they are clearly inappropriate. (Their 2SD point below mean frequently falls below zero.)

To compare groups adequately the same pooled tumour must be injected into the same batch of mice. Numbers obtained from one pool can frequently be about 20 mice, but sometimes less. These

limit the number of parallel experiments.

Statistics

Mann Whitney 'U' Test was employed with results expressed as mean and range.

Pulmonary Metastases

Obviously pulmonary metastases kill, but some drugs, for example Heparin, may reduce pulmonary metastases but increase metastases to other sites. To show this would entail the invidious task of analysing other significant organs - such as the liver - for metastases. I did not have the expertise to indulge in total body histology; I tackled the problem by relating results in pulmonary metastases to life span (though any mouse which was ill was culled). These results were analysed on an acturial basis.

Low Immunogenicity

Two methods were used to examine the claims that this model has a low immunogenicity.

1. Tumour formation and metastases in outbred mice.

Pooled tumour was injected either into C57 Black mice or a variety of outbred mice. The incidence of primary takes and the frequency of metastases in those with primary was found.

Four experiments were performed as demonstrated below. There was a significant difference with both the incidence of primary takes, and numbers of metastases. This suggested that sarcoma cells are antigenic.

			Incidence of Primary Takes	Metastases	
1.	C57	n = 10	9	5.5	(0-9)
	Outbred	n = 10	5	3	(0-6)
2.	C57	n = 12	12	9	(3-21)
	Outbred	n = 12	7	2	(0-9)
3.	C57	n = 8	7	7.5	(2-14)
	Outbred	n = 8	2	0	
4.	C57	n = 6	6	9	(6-13)
	Outbred	n = 6	0		

2. Repeated injections of C57 Sarcoma.

It might appear simple to show that in those mice who had failed to take a primary innoculation that further injection would fail to alter this state. Unfortunately there is a fallacy in this argument; if the innoculation has already failed this may be due to reasons other than the immune system; further innoculations would fail again.

For these reasons tumour cells - in suspension - were killed by heating and then injected. This was repeated weekly x 3. Live tumour was then innoculated and also into a control group who had been given injections of normal mouse liver cells.

The incidence of takes was not significantly different in each separate experiment but was overall ($p < 0.05$). In the three experiments asterisked there was a significant reduction of metastases ($p < 0.05$) and this difference existed overall ($p < 0.01$).

			Incidence of Primary Takes	Metastases	
1.	C57 control	n = 11	10	7	(1-15)
	Pre-innoc	n = 11	9	4	(0-6) *
2.	C57 control	n = 7	7	13	(4-24)
	Pre-innoc	n = 7	6	6	(0-11) *
3.	C57 control	n = 12	12	9	(2-19)
	Pre-innoc	n = 12	10	6	(2-14)
4.	C57 control	n = 8	7	11	(8-27)
	Pre-innoc	n = 8	5	7	(4-13) *

Conclusion

The tumour has the ability to produce an immune response.

Levamisole

This antihelminthic agent is known to induce in man an improved cellular immune response when previously impaired. The mechanism is debated but may be mediated via the macrophage. In the mouse the results are more questionable partially depending on dosage and routes of administration. I chose to give Levamisole over three days, subcutaneously, (1mg/Kg) prior to a mock laparotomy outlined previously. Controls were chosen randomly (coin spinning) and given isotonic saline injections.

The four experiments only examined the number of metastases; measurement of primary tumours revealed no difference between the groups. As can be seen in the table there was a significant difference achieved ($p < 0.05$) in three of the experiments, and in the fourth group whilst not statistically different the Levamisole group had less

metastases. The overall probability was also significant.

Results

Experiments		No. of Mean	Metastases (Large)	Significance
1. Levamisole	n = 11	7.5	(3-13)	$p < 0.05$
Control	n = 12	13.5	(9-22)	$p < 0.05$
2. Levamisole	n = 8	3.5	(0-5)	$p < 0.05$
Control	n = 8	5	(2-6)	
3. Levamisole	n = 6	4.5	(0-6)	$p < 0.05$
Control	n = 6	8	(4-13)	
4. Levamisole	n = 9	5	(2-11)	N.S.
Control	n = 8	6.5	(2-11)	

Discussion

The effect of Levamisole, or any other immune stimulant would theoretically be limited in any situation where tumour cells were in abundance. This experiment was a severe test of the drug, which was effective; presumably by an enhanced cellular response.

Conclusion

Although the inherent defect in using an animal model remains, several specific criticisms to the C57 Black Lewis Lung sarcoma model have been answered. The tumour does contain antigenic sites, which can be recognised by either foreign mice or C57 Black; modulation of the immune response with Levamisole appropriately decreased tumour metastases.

Statistical analysis was possible using non parametric tests in each small group derived from the same tumour pool.

CHAPTER 3

HEPARIN, DEXTRAN AND COUMARINS

Heparin, a mucopolysacchride composed of sulphated D-glucosamine and D-glucoronic acid, is heterogeneous; estimates of molecular weight range from 600 to 20,000. It contains a large number of O- and N- sulphate linkages and is strongly acidic. Hydrolysis of the ester linkage results in an altered molecular shape and lost activity. The anticoagulant effect may be due to reaction with anionic groupings on clotting factors to produce complexes. Following intravenous injection Heparin is evenly distributed between white cells and plasma; it disappears exponentially but at a rate dependent upon the dose; it is partially metabolised by the liver to a weakly active uroheparin.

The effect of Heparin on tumour metastases is well known; the frequency of tumour lodging in the pulmonary vascular bed is decreased. As I have already mentioned, this may result in increased peripheral metastases. This effect appears to be due to either an action preventing fibrin formation around the tumour cells or directly altering the adhesive quality of the tumour cell.

Several reasons suggest an immunological role for Heparin. It blocks the eosinopaenic response to insulin, corticosteroids and ACTH; it inhibits the mixed lymphocyte reaction (Currie 1967), and activates the spleen cells of mice of several species (Ruhl et al 1974). It produced lymphocytes when given to a calf.

The possibility of an immunological effect is enhanced by the chemical similarity with known B cell mitogens: dextran sulphate; dextrans; polyethylene sulphate; poly A- poly U; poly I - poly C.

Polymers, especially anionic polymers, are usually active, though dextran phosphate is an exception.

I decided to explore the possibility of immunological effects in the leucocyte migration test and upon lymphocyte transformation.

Effect of Heparin upon Leucocyte Migration

The method was as previously described, but with blood anticoagulated by defibrination. The Heparin used was made by Weddel (preservative free). Four consecutive experiments were initially employed to discover the effect of Heparin. Control migrations were compared with a dose response; Heparin 0.01 units/ml to 100 units/ml; each were adjusted to the control pH.

TABLE Dose Response of Heparin; Effect upon Leucocyte Migration

(Area mm^2, mean - range; 4 experiments)

		Mean (mm^2)	Range
Control		97.5	85 - 112
Heparin:	0.01 unit/ml	98.2	88 - 121
	0.1 unit/ml	95.6	81 - 116
	1 unit/ml	102	91 - 125
	10 unit/ml	111	99 - 141
	100 unit/ml	129	118 - 145

There was a significant increase in area migrated at 1 and 10 units/ml. To explore the effect further two concentrations of Heparin were chosen; 0.01 unit/ml and 1 unit/ml. I showed that these had no toxic effect on lymphocytes, by trypan blue exclusion.

Migrations were performed upon leucocytes from Heaf grade 2 positive subjects using control, control and PPD, Heparin, Heparin and PPD.

TABLE The Effect of Heparin on PPD Induced Inhibition of Leucocyte Migration; Results in mm^2, mean and range of 4 experiments on one subject.

	Mean (mm^2)	Range
Control	99.75	83 - 115
+ PPD	71.2	64 - 79
Heparin 0.1 unit/ml	100.22	85 - 111
+ PPD	70.0	61 - 82
Heparin 10 unit/ml	115	102 - 155
+ PPD	106	94 - 131

The previous effects of Heparin alone were confirmed. Significant inhibition of migration occurred in all four experiments on control leucocytes exposed to PPD; only in one experiment did PPD induce significant inhibition when in the presence of Heparin at 10 units/ml. Heparin at 0.1 unit/ml produced no significant effect on the PPD induced inhibition.

I then performed an experiment to find the site of action of this effect. I used the two stage version of the leucocyte migration test. Lymphocytes were initially incubated with PPD to produce lymphokine(s), which were then tested upon predominantly lymphocyte free leucocyte migrations. PPD was always added to the control migration. To aliquots of the lymphokine containing supernatants I added 0.1 unit/ml and 10 units/ml of Heparin.

TABLE Two Stage Migrations: Means (mm^2) and ranges of 5 experiments on the same Heaf grade 1 positive subject; the effect of Heparin

	Mean (mm^2)	Range
Control supernatant	125	92 - 141
+ LIF	82.75	67 - 95
Heparin 0.1 unit/ml	131	97 - 135
+ LIF	84.1	73 - 101
Heparin 10 units/ml	152	129 - 177
+ LIF	157	135 - 182

Heparin at 0.1 u/ml had no discernable effect. At 10 u/ml it produced exaggerated migration and prevented inhibition of migration.

Conclusion

Heparin interferes at a high concentration with leucocyte migration inhibition to antigen. Probably LIF release occurs as usual but the decreased motility and increased adherence found after exposure to the antigen is anulled. Even the control migrations, possibly influenced by the FCS, were increased. I suspect that this is a simple Heparin membrane effect, similar to that suggested for tumour cells.

Effects of Heparin upon Lymphocyte Transformation

It was already clear that Heparin should effect changes in lymphocyte transformation if it were to behave like the other members of the sulphated polysaccharide group. I was worried by the

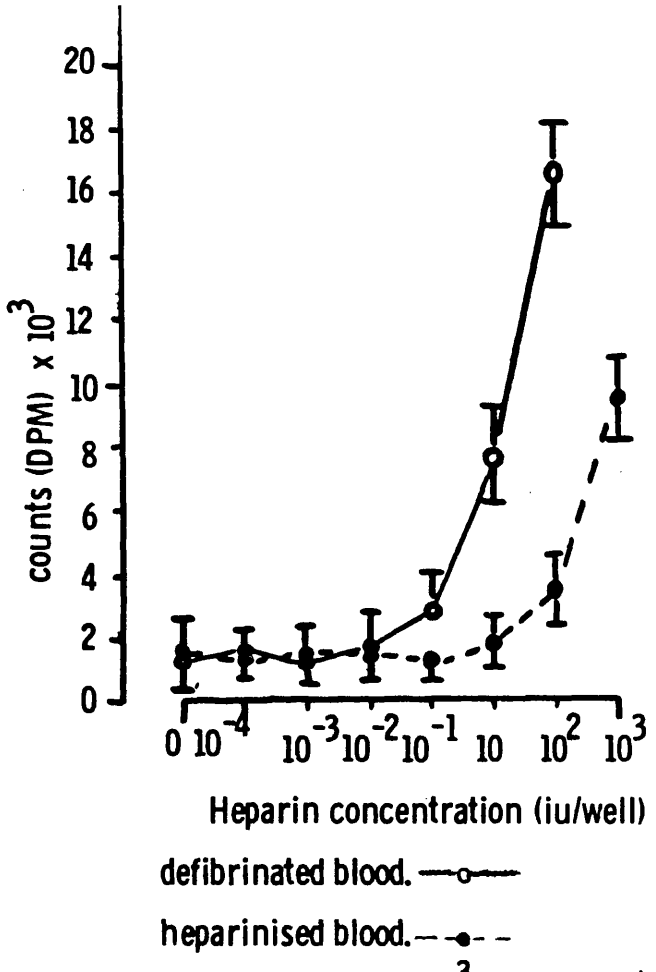


Fig. Effect of heparin upon H^3 Thymidine incorporation in heparinised and defibrinated blood. 48 hours incubation. Means ± 1 SE of 8 experiments involving 4 subjects.

consequence of this postulate; Heparin is widely used in diverse immunological methods even by the purists such as Coutinho & Moller.

I used the whole blood method, but the blood was anticoagulated by defibrination.

Result

Heparin increased activity significantly at 5 iu/ml ($p < 0.05$) and 50 iu/ml ($p < 0.01$); see figure.

I had shown that Heparin produces lymphocyte transformation. In defibrinated blood, fibrinogen and the coagulation cascade are missing, this may be the reason why this effect of Heparin is not often seen. I repeated the above experiment in whole blood, anticoagulated with Heparin, or by defibrination. To half of the defibrinated blood I added fibrinogen (Human, Searle) to a final concentration of 3g/L and Heparin 2.5 units/ml.

As can be seen in the figure the dose response was shifted to the left in the presence of fibrinogen, which was similar to the results in whole blood anticoagulated with Heparin. Substimulatory doses of mitogens can have effects, but this also depends on which type of cell is stimulated. Preponderantly pure B and T cell populations were prepared from defibrinated blood. PHA; dextran sulphate; and Heparin (5000 iu/ml) were incubated with aliquots of the preparations. The results of the four experiments are shown in the Table.

TABLE Effect of Heparin on B or T Cell Populations; compared with known mitogens

		Means ± SEM
B cell	Control	15,005 ± 3,250
	PHA	17,100 ± 4,109
	Dextran Sulphate	22,222 ± 6,158
	Heparin	21,195 ± 5,971
T cell	Control	12,175 ± 3,157
	PHA	95,431 ± 9,758
	Dextran Sulphate	13,977 ± 2,210
	Heparin	11,299 ± 2,911

Heparin, like Dextran Sulphate, had no significant effect in the preponderantly T cell preparation, but had a significant ($p < 0.01$) effect in the B cell preparation. This is strongly suggestive that Heparin is a B cell mitogen. If so it might interfere with other mitogens. Whole blood, defibrinated, was divided into aliquots. To the first series a Heparin dose response was performed; to the second PHA was added 12 hours after the start of the incubation; to the third a Heparin dose response was performed and PHA was added 24 hours later; to the fourth Heparin and PHA were added simultaneously; to the fifth PHA alone was added; to the sixth Heparin was added 24 hours after the start of the incubation, and finally PHA was added immediately, Heparin 24 hours later.

TABLE Interference of Heparin with PHA Induced Stimulation of Thymidine, DPM, Means ± ISE, 4 experiments

Control	PHA (added 12h after start of incubation)
1650 ± 189	20,568 ± 2150
Heparin	Heparin:48hrs PHA (added after 12h)
a) 0.5 iu/ml 1,890 ± 211	a) 0.5 iu/ml 18,987 ± 2131
b) 5 iu/ml 2,750 ± 235	b) 5 iu/ml 11,150 ± 1988
c) 50 iu/ml 8,176 ± 614	c) 50 iu/ml 9,859 ± 1750
PHA:48 hrs	
81,013 ± 6459	
PHA + Heparin:48 hrs	
a) 0.5 iu/ml 84,048 ± 7145	
b) 5 iu/ml 88,150 ± 5298	
c) 50 iu/ml 118,345 ± 4117	
Heparin (added after 12hr)	PHA(48h) & Heparin (added after 12hr)
a) 0.5 iu/ml 1,519 ± 214	a) 0.5 iu/ml 79,178 ± 6284
b) 5 iu/ml 1,741 ± 199	b) 5 iu/ml 83,310 ± 7145
c) 50 iu/ml 4,145 ± 288	c) 50 iu/ml 82,219 ± 6946

Heparin significantly interferes with PHA stimulation if added prior to the PHA; PHA similarly interferes with Heparin. This effect is best seen at 5 units/ml when Heparin produces little effect on its own, but can decrease PHA stimulation.

Conclusion

Heparin is a lymphocyte stimulant, acting preponderantly on B cell. Interference with other mitogens can occur.

Immunological Effects of Dextran 70 and Carragheenan

An elegant way of investigating the effects of Heparin is to examine the related chemical structures of Dextran and Carragheenan. Any data collected separately on Dextran would be useful because of the clinical usage; Carragheenan (Lambda; Marine Colloid Inc) that ubiquitous Irish bog moss is not only widely used as an expander and surfactant in foodstuffs and drinks but has been used immunologically.

Dextran

This simple polysaccharide has anticoagulant properties by interfering with fibrin cross linking. Early preparations produced occasional anaphylaxis; high molecular weight Dextrans evoke a B cell transformation. Unlike Heparin it is not highly anionic and has minimal membrane effects.

Leucocyte Migration

I repeated the experiments performed on Heparin but using Dextran instead.

Unlike Heparin, Dextran did not produce any enhancement of migration; the high concentration produced some impairment ($p < 0.05$) of area migrated. This concentration produced greater lymphocyte death (dye exclusion) than in the controls. The effect on migration

was probably due to toxicity. When added to PPD Dextran had no appreciable effect.

TABLE Dose Response of Dextran on Leucocyte Migration

	Area Migrated (Mean) mm^2	Range
Control	107.8	89 - 125
Dextran 1	106.9	85 - 121
2	104.75	80 - 127
3	105.77	82 - 126
4	88.9	75 - 98

TABLE Effect of Dextran on PPD Induced Migration Inhibition of Leucocytes

	Area Migrated (Mean) mm^2	Range
Control	104.5	87 - 121
Dextran	107.8	83 - 125
PPD	69.7	54 - 82
PPD - Dextran	73.1	56 - 84

Conclusion

These results suggest that the important effect of Heparin in the LMT is by changing the cell membrane rather than a role of anticoagulant.

Transformation

I explored the effect of Dextran on lymphocyte transformation in a similar way to that of Heparin. I enclose an off print from the paper published (Burden et al, 1977).

When this preliminary publication was printed we were unsure which lymphocyte population was stimulated. Since then I have confirmed that like Heparin Dextran affects B cells.

TABLE Effect of Dextran on B and T Cells, Compared with PHA and Dextran Sulphate

		Means	- SEM
B Cell	Control	14,975	4,119
	PHA	16,035	3,988
	Dextran Sulphate	24,175	5,239
	Dextran	28,229	4,987
T Cell	Control	12,275	4,880
	PHA	86,594	11,179
	Dextran Sulphate	11,199	3,141
	Dextran	12,850	4,570

Conclusion

The presence of sulphate residues are not needed to produce transformation. Perhaps conformational requirements - from folding of the polymer - are the most important. I would suggest that all polysaccharides are capable of inducing transformation although the greater the chain length and the more free sulphate (or anionic)

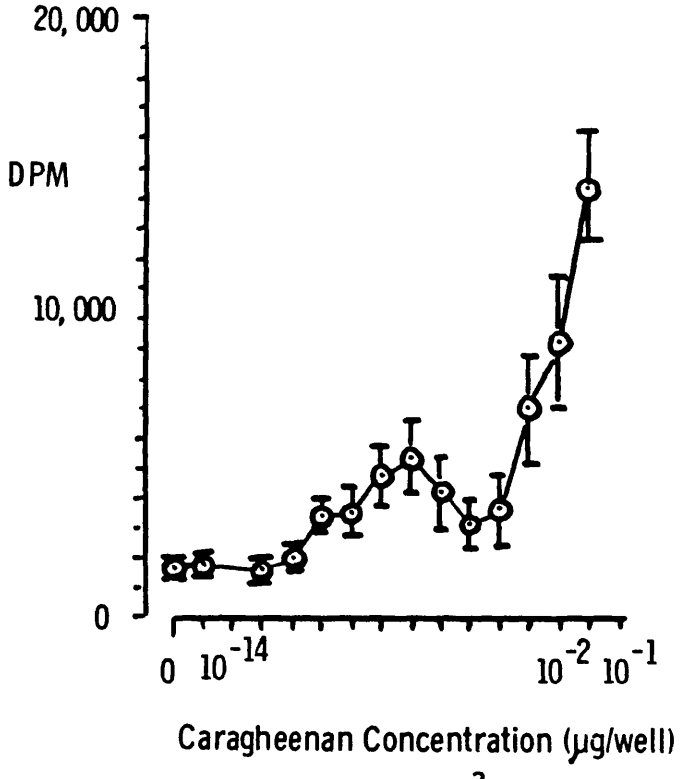


Fig. Effect upon thymidine H^3 incorporation in D. P. M. of Caragheenan, 48 hour incubation, Mean + 1 SE of 12 experiments, involving 6 subjects.

radicals the greater the effect.

The cells affected are B cells; antibody may be produced and certainly is in response to some Dextrans and Dextran Sulphate.

Carragheenan

To extend these conclusions further, I chose to investigate the potential of Carragheenan to produce lymphocyte transformation. This substance is well known to clinicians from when it was claimed to have curative properties for peptic ulcers; to pharmacologists because s.c. injection of Carragheenan is a reproducible method to produce inflamation; and to immunologists because of its known ability to kill macrophages (Allison et al, 1966) and to enhance graft survival (Everson et al, 1977). Most work on lymphocytes have suggested that it kills or just interferes with PHA response (Lake et al, 1971). Some though have observed increased numbers of nucleated spleen cells in the mouse following intravenous injection (Thompson et al, 1976). I performed experiments to find the effect of Carragheenan on whole blood (anticoagulated by defibrination). Dose responses were studied by making titre dilutions of the polysaccharide, using 16 replicates for each dilution. The results showed a 2 peak dose response (Figure) with significant increases occurring from 5×10^{-10} ug/ml to the maximum concentration of 5×10^{-1} ug/ml (p 0.01).

I concluded that Carragheenan was a lymphocyte mitogen. Was it active on B cells?

TABLE Effect of Carragheenan on B or T cells, compared with PHA and Dextran Sulphate (*significant $p < 0.05$)

		Means	±SEM
B cell	Control	12,667	3,715
	PHA	13,143	2,917
	Dextran Sulphate	25,219	7,143
	Carragheenan	28,173*	6,288
T cell	Control	13,154	4,005
	PHA	91,148	10,977
	Dextran Sulphate	12,177	3,450
	Carragheenan	13,298	3,788

Like Heparin and Dextran, Carragheenan interfered with PHA response, depending upon the time of presentation.

TABLE Effect of Carragheenan upon response to PHA, Whole Blood Method

Control	PHA (added after 24h)
1,719 ± 203	22,158 ± 2300
Carragheenan	**PHA (added after 24h & Carragheenan)**
a) 5×10^{-11} ug/ml 1823 ± 311	a) 9,008 ± 1744
b) 5×10^{-7} ug/ml 2791 ± 229	b) 2,110 ± 581
c) 5×10^{-2} ug/ml 8145 ± 640	c) 9,987 ± 1779
Carragheenan + PHA	**PHA for 48h**
a) 5×10^{-11} ug/ml 89197 ± 9403	88,647 ± 8063
b) 5×10^{-7} ug/ml 95551 ± 8983	
c) 5×10^{-2} ug/ml 97491 ± 9138	

TABLE Con/d..

Carragheenan (added after 24h)	PHA + Carragheenan (added after 24h)
a) 5×10^{-11} ug/ml 1916 ± 271	a) 87,114 ± 8193
b) 5×10^{-7} ug/ml 1788 ± 310	b) 89,218 ± 8801
c) 5×10^{-2} ug/ml 1865 ± 289	c) 89,987 ± 9117

I believe that an alternative explanation for immune tolerance to grafts following Carragheenan may be the production of anti idiotypic antibodies. It is not impossible that this production may be increased further in the presence of defective macrophages. These results are not in conflict with the previous findings of Lake et al despite their statement that Carragheenan was not a stimulant, because on reappraisal of their data, at a concentration of 125 ug/ml there was a significant increase in thymidine incorporation ($p < 0.05$).

Animal Experiments

I decided to limit these to some interesting points, rather than to repeat whole experiments by previous workers. It firstly seemed reasonable to check that subcutaneous Heparin would affect the frequency of metastases. Heparin 3 unit/gram was given subcutaneously daily in divided doses to randomly chosen C52 black mice bearing tumour. It was given for three days before resection until the time of culling.

TABLE Effect of Heparin on Frequency of Metastases

		Mean Numbers of Metastases	Range	Significance
Control	n-9	6.7	0 - 14	$p < 0.05$
Heparin	n-8	3.1	0 - 5	
Control	n-8	15.5	7 - 24	$p < 0.05$
Heparin	n-9	10.9	6 - 14	
Control	n-6	6.8	0 - 12	NSD
Heparin	n-6	5.3	0 - 9	
Control	n-7	8.1	0 - 12	NSD
Heparin	n-7	6.4	0 - 9	

Cumulative probability p 0.001

This effect, whilst not marked, was significant in two experiments, and the cumulative probability was highly significant. Having demonstrated this I determined the effect upon survival.

TABLE Effect of Heparin on Post Resection Survival

		1 week	2 weeks	3 weeks
Control	n-6	6	6	3
Heparin	n-6	5	4	0
Control	n-5	5	4	2
Heparin	n-5	5	3	2
Control	n-6	5	5	2
Heparin	n-6	6	5	3
Control	n-4	4	4	1
Heparin	n-4	3	2	0

This showed no significant alteration of survival in the Heparin treated group. In case this was due to Heparin toxicity, I gave Heparin at this dosage to a group of 5 normal animals for four weeks, and compared the results with a peer group; there was no difference in size or survival; in fact all survived.

The conventional explanation for this effect, would be that the Heparin, by acting as an anticoagulant had decreased the frequency of pulmonary metastases but increased the frequency of non pulmonary metastases. Another explanation could be that the Heparin was acting as a B cell stimulant evoking either interference with T cell tumour cell killing, or producing an enhancing serum effect.

Coumarin Anticoagulants

The oral anticoagulants have no direct action on circulating clotting factors, but block the hepatic formation of II, VII, IX and X by competitively inhibiting the action of vitamin K. Two sites of action of vitamin K have been suggested (Lowenthal & Macfarlane, 1967; Lowenthal & Birnbaum, 1968), one is readily blocked by the coumarins; the other, requiring high concentrations of vitamin K, is coumarin insensitive.

The coumarins cover a wide range of structures, and although the drug I used for in vitro experiments (Bishydroxycoumarin) is different from Warfarin it shares the same derivation, and the seemingly minimum requirement of an intact 4-hydroxycoumarin residue with the 3 position substituted by a carbon residue or hydrogen atom (Kralt & Claasen, 1972).

As I have already mentioned, much work has been conducted upon Warfarin and its clinical effects upon metastases. It is thought to have two actions; one as an anticoagulant; the other as an inhibitor of mitosis. The drug levels achieved in one trial (Thornes, 1974) were only sufficient to produce a minimal change in prothrombin time but reduced the incidence of metastases. This suggested that the action was not related to anticoagulation. I simply examined the effects in vitro upon lymphocyte migration and lymphocyte transformation.

Leucocyte Migration

A dose response for Bishydroxycoumarin in the LMT was used, dilutions from 10^{-10} M being tested in control and PPD containing media.

TABLE Bishydroxycoumarin and the LMT : Dose Response

3 experiments on heaf subjects

Bishydroxycoumarin concentration	Control Migration Area		PPD Migration Area	
	Mean	Range	Mean	Range
0	112.45	92-133	87.77	72-98
10^{-10}M	113.78	93-138	89.15	68-104
10^{-8} M	110.89	81-129	105.58	83-111
10^{-6} M	110.61	87-133	106.12	84-122
10^{-4} M	101.66	82-115	98.63	80-124
10^{-2} M	96.77	76-116	97.71	73-128

These results show that at 10^{-8} M the significant inhibition of migration due to PPD is ablated; higher concentrations decrease the

control migrations. This concentration is that seen during clinical usage (with Warfarin) occasionally it provokes interference with hepatic oxidative-reduction enzymes; and decreases hypersensitivity reactions.

Lymphocyte Transformation

A similar experiment was performed on the effect of the coumarin on lymphocyte transformation. Four subjects, all gave venous blood anticoagulated by defribrination. Dose responses were undertaken of Bishydroxycoumarin from 10^{-10} to 10^{-6}M on control and PHA stimulated cultures.

TABLE Effects of Bishydroxycoumarin on Lymphocyte Transformation

Warfarin Concentration	Mean ± SEM Control	Mean ± ISEM PHA
0	2210 ± 211	93567 ± 9122
10^{-10}M	2180 ± 196	97170 ± 8989
10^{-8} M	2318 ± 179	95159 ± 9941
10^{-6} M	1770 ± 113	81180 ± 11150

Significance $p < 0.05$ compared with control

The results showed a significant effect at 10^{-6}M, reducing the PHA count, but not at 10^{-8}M the concentration at which the migrations were significantly affected.

I suspect that this represents differences between cells. Viability studies showed significant killing of lymphocytes at 10^{-6}M, but not at lower concentrations.

To resolve this problem I tried the effect of the coumarin on the two stage LMT; generating lymphokine normally and testing this on coumarin medium containing leucocytes.

TABLE Effect of Bishydroxycoumarin on Leucocyte Migration Inhibition to Lymphokines

Bishydroxycoumarin Concentration	Migration Area Mean	Range
0	65.8	41 - 94
10^{-10}M	64.1	40 - 98
10^{-8} M	93.2	75 - 118
10^{-6} M	98.1	79 - 127

This showed a close similarity with the results of the one stage. Significant reduction of the lymphokine induced inhibition of migration occurred at 10^{-8}M. It would seem that PMNL are more sensitive to the cytocidal effects of the coumarins than are lymphocytes.

Conclusion

The coumarins produce inhibition of leucocyte migration, and decreased lymphocyte transformation. This at high concentrations, and cytocidal effects on various cells are probably seen at differing concentrations, with lymphocytes more resistant than PMNL.

CHAPTER 4

FIBRINOGEN AND FIBRIN DEGRADATION PRODUCTS

Most work on the immunological properties of fibrin degradation products (FDPs) has alternated between an awareness of the in vivo effects of fibrin, as shown by Barnhart in 1968 using the skin window technique in dogs, and the descriptions of alterations in chemotaxis of leucocytes to a variety of fibrinogen degradation products. Clot supernatants prepared by the action of thrombin on fibrinogen are chemotactic for neutrophils, and eosinophils; unlike either thrombin or fibrinogen (Kay et al 1973). This activity appears to be due to fibrinopeptide B: shown by progressive purification, the effects of Contortrix or Arvin venoms, and the activity of synthetic fibrinopeptide B (Kay et al 1974). Chemotactic activity for human neutrophils and eosinophils has been shown by the action of plasmin on human fibrinogen (McKenzie et al 1975; Kay & Kaplan 1975).

I decided to explore the effects of FDPs on leucocyte migration inhibition because of the similarities between this and leucocyte chemotaxis, and because of the known difficulty in standardisation of the LMT with variability resulting from different batches of serum (Ling 1976). I assessed the effects initially using the conventional leucocyte migration test, and then explored the site of action by using a two stage leucocyte migration, and the whole blood technique for detecting lymphocyte transformation.

Materials and Methods

1. Preparation of FDPs

Human fibrinogen was dissolved in phosphate buffered saline

(PBS-) to a concentration of 20mgms/ml. Thrombin (Bovine, Sigma) 20 iunits/ml of fibrinogen solution was added and the fibrinogen allowed to clot. The clot was squeezed manually to express supernatant, washed x3 in PBS and finally PBS added to give the initial volume. Porcine plasmin (Sigma) was added (20 ug/ml) and the fibrin and plasmin incubated for 24 hours at 37°C. The supernatant was then removed and incubated for a further 24 hours then assayed for plasmin using the fibrinolytic assay. This showed a plasmin concentration of not more than 0.02 ug/ml (the limit of the assay). The concentration of FDPs was found by subtracting the dry weight of fibrin following plasmin incubation from the dry weight before incubation.

Leucocyte Migration

Blood was taken from 6 volunteers, anticoagulated with Heparin and prepared as previously described. To the migration wells were added a dose response of FDPs: 10, 20, 40 and 80 ug/ml.

TABLE Effect of FDPs on Leucocyte Migration, mean of 6 separate experiments

Concentration of FDPs ug/ml	Migration Area mm^2 Mean	(range)
0	109	(87-145)
10	95	(78-139)
20	84	(66-118)
40	68	(60- 77)
80	67	(58- 74)

There was a significant effect of the FDPs, with a plateau at about 40 ug/ml. To see if synergism occurred between the FDP effect and the action of PPD an experiment was performed using a concentration of FDP which did not produce significant inhibition (10 ug/ml) was obtained from 4 volunteers anticoagulated with Heparin and the leucocytes separated. Each was divided into 4 aliquots. One acted as a control, one had 10 ug/ml of FDP added, one PPD 10 ugm/ml and to one both FDP 10 ugm/ml and PPD 100 ugm/ml was added.

TABLE Means and ranges of 4 experiments on Migration Inhibition to FDP and PPD

	Control	FDP 10 ug/ml
Control	113 (87-134)	98 (78-127)
PPD 100 ugm/ml	85 (65-101)	72 (59-85)

The wells with both FDP and PPD present produced more inhibition than either FDP or PPD, this was significant ($p < 0.05$ U test). Since FDPs have been shown to have direct effects upon leucocytes I conducted an experiment using the two stage technique to demonstrate that LIF can be released with FDP. Lymphocytes were obtained, to one aliquot was added FDPs (80 ug/ml); the other acted as control. Following a 24 hour incubation, the lymphocytes were removed and to the control supernatant FDPs added to 80 ug/ml concentration. The supernatants, adjusted for pH, were added to migration wells and lymphocytes deplete PMNL allowed to migrate.

TABLE FDP (80 ug/ml) Induced Lymphokine Release, and Effect on PMNL Migration Area. Mean of 5 experiments.

	Migration Area	
	Mean	Range
FDP incubated supernate	85	(61-101)
Control supernate	116	(97-144)

There was a significant reduction in migration area (p 0.05 U test). These experiments showed the FDPs release LIF.

Effects of FDPs on Lymphocyte Transformation

Whole blood, anticoagulated with Heparin, was diluted as previously described and dispensed into the microlitre wells. A FDP dose response was performed. The table shows the mean of 4 experiments on separate volunteers.

There is a dose response, significantly enhanced thymidine uptake occurring at 40 ug/ml ($p < 0.05$) and 80 ug/ml. I then attempted to determine if the FDPs could interfere with PHA response - analogous to the dextran findings.

TABLE Dose Response FDPs and Lymphocyte Transformation

	DPM Mean	± ISD
Control	8450	921
FDP 10 ug/ml	8541	945
20 ug/ml	9136	879
40 ug/ml	9554	968
80 ug/ml	11149	971

I chose 20 ug/ml for the concentration of FDP : it probably caused stimulation but in any one experiment performed previously it had not done so significantly. Whole blood, anticoagulated with Heparin, was diluted and divided into aliquots.

TABLE Effects of FDPs on PHA Transformation

	Incubation Time	Mean ± SD
Control	48 hr	7961 ± 710
FDP	48 hr	8432 ± 765
FDP added after 24 hr	48 hr	8210 ± 732
PHA	48 hr	156982 ± 33410
PHA added after 24 hr	48 hr	81791 ± 21149
PHA) FDP)	48 hr	177714 ± 35142
FDP (48hr) PHA (added after 24 hr)	48 hr	127845 ± 29113
PHA (48 hr) FDP (added after 24 hr)	48 hr	149487 ± 30157

The work showed the FDPs if added before PHA produced greater thymidine incorporation than PHA alone. If they were added after the PHA, counts were similar to PHA alone.

Conclusion

Fibrin degradation products provoke lymphokine release and lymphocyte transformation. They exaggerate PPD induced inhibition of leucocyte inhibition and PHA induced lymphocyte transformation.

The Effect of Fibrinogen

Human fibrinogen (Searle) was added to whole blood, anticoagulated by defribrination, to which had been added Heparin 2.5 units/ml. A dose response of fibrinogen was performed, using the effectible blood concentrations of 0.1, 1, 2, 3, 4 and 5 G/L. Any greater concentration provoked clotting.

TABLE Fibrinogen and Thymidine Incorporation, Means of experiments on 9 volunteers, not previously transfused.

	Mean	±ISD
Control	7678	729
Fibrinogen 0.5	8315	815
1	8101	770
2	7510	730
3	7514	729
4	7495	781
5	7999	914

The lowest concentration, 0.5G/L produced a slight increase in counts, which I think was due to the Heparin effect reported earlier; at the highest concentration, 5G/L, there was sporadic high counts influencing the variance, this was due to poor washing of the viscid cell-fluid mixture through the filter paper. It seemed reasonable to suspect though that the concentration of fibrinogen 1G/L and 4G/L could be compared; and such comparison would be due to the fibrinogen and not Heparin. I chose volunteers who had never received transfusions to obviate any who may have been exposed to foreign fibrinogen.

The Effect of Fibrinogen on PHA Transformation

Whole blood, defibrinated, anticoagulated with Heparin 2.5iu/ml, was divided into two aliquots after dilution. Fibrinogen was added to give whole blood concentrations of 1 and 4G/L. The aliquots were then distributed into microtiter wells and incubated for 2 days. To these preincubated wells was added either PHA or an equal volume of isotonic saline, and the wells incubated for another 48 hours.

TABLE Effect of Fibrinogen on PHA Induced Transformation

	Fibrinogen 1G/L	Fibrinogen 4G/L
Control	6915±697	7141±669
PHA	157315±31665	98142±21410

The table represents the means and ± ISD of 9 experiments on separate individuals; each experiment demonstrated the same finding

that PHA transformation was depressed with the higher fibrinogen level.

Discussion

To ensure that this effect was simply due to fibrinogen is difficult. I have shown that Heparin can interfere with immune responses and that the amount of 'free' Heparin is proportional to the amount of fibrinogen. I have previously shown that if Heparin and PHA are added simultaneously then the effect of Heparin - if anything- is to stimulate. Fibrinogen could itself provoke transformation, this was not found in the subjects examined.

The cells were allowed to reside in their different fibrinogen environments for 48 hours before the stimulants were added, on the simple basis of allowing time for any 'coating' effect of fibrinogen to occur. I repeated the experiments without the 'coating' incubation and no difference was found between the two groups.

I believe that this simple concept is reasonable. At high concentrations of fibrinogen a 'coat' could develop, preventing either ligand attachment or any conformational membrane change required for transformation.

Conclusion

Fibrinogen interferes with lymphocyte transformation, possibly by interference with ligand binding.

Epicardial ST-segment Recording

The degree of epicardial ST-segment elevation provides an independent index of myocardial ischæmic injury.[10] This is recorded from five disc electrodes (1·5 mm diameter) sutured to the epicardium at least 1 cm from the border area of ischæmia in the potentially ischæmic zone. By passing the electrograms through an averaging switch-box, a signal averaged from the input of five leads is obtained, and this may be recorded either from the oscilloscope or subsequently from tape. Constant heart-rate must be maintained by atrial pacing to eliminate rate-dependent influences.[11]

RESULTS

A characteristic sequence of recordings following coronary-artery occlusion in a dog is shown in fig. 1, the cardiac action potential being recorded from the centre of the ischæmic zone. The notch on the upstroke of the action potential corresponds to the "extrinsic potential" of Prinzmetal et al.[8] and is related to the activation of tissue between the recording and indifferent electrodes.[9] By recording differentially from two closely applied floating microelectrodes, large extrinsic potentials are eliminated. Endocardial activation can be seen to precede epicardial activation. After coronary occlusion action-potential configuration changes rapidly, with shortening, loss of amplitude, and disappearance of the initial rapid phase of depolarisation. Changes of the order of those seen in isolated superfused anoxic ventricular muscle after 30 min of total anoxia are observed in vivo after 2–3 min.[12]

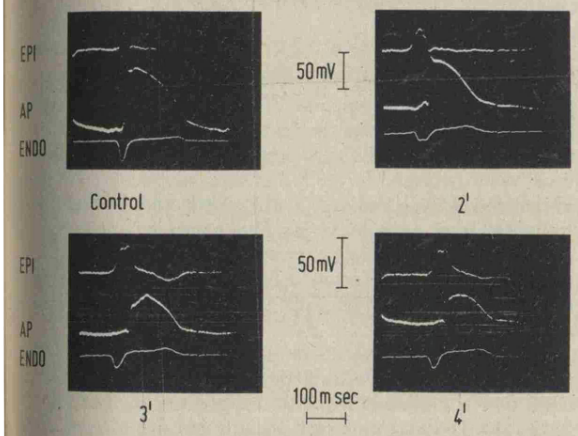


Fig. 1—Recordings of action potential (AP) endocardial (ENDO), and averaged epicardial (EPI) electrograms from dog heart in situ before and 2, 3, and 4 min after coronary-artery occlusion.

Note shortening of the action potential and progressive delay in its onset with respect to the endocardial-potential loss of the early rapid phase of depolarisation. The epicardial electrogram demonstrates the conduction abnormality and ST-segment elevation.

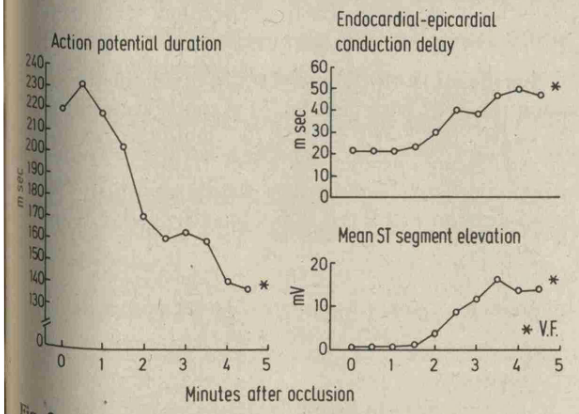


Fig. 2—Changes in action-potential duration, endocardial-epicardial conduction delay, and mean ST-segment elevation in dog heart after acute coronary-artery occlusion.

Ventricular fibrillation (V.F.) resulted after 4 min 50 s of ischæmia, at a time of maximum recorded conduction delay and action-potential shortening.

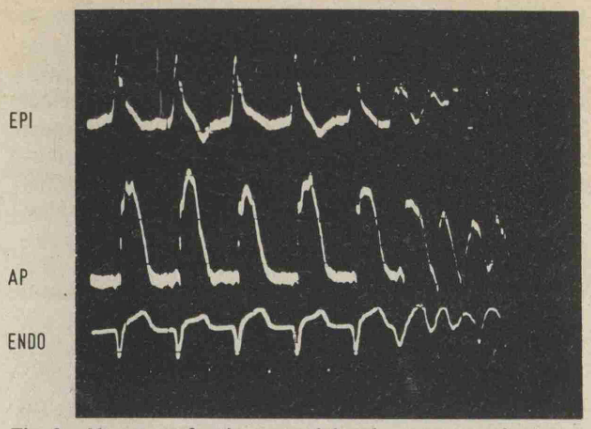


Fig. 3—Alternans of action potential and ST-segment of epicardial and endocardial electrograms preceding ventricular fibrillation.

Note the increased asynchrony between intracellular and extracellular recordings at onset of fibrillation.

More detailed findings from one dog are shown in fig. 2, in which ventricular fibrillation resulted after $4\frac{1}{2}$ min of ischæmia. Within 30 s of occlusion, transient prolongation of action potential occurred and was followed by shortening and development of a more triangular shape. By 2 min endocardial-epicardial conduction was delayed, and mean epicardial ST-segments were elevated. At 4 min after occlusion, the action potential assumed an appearance similar to that of "slow response" potentials.[13] Such potentials occur with a reduction in transmembrane resting potential to less than −60 mV, at which voltage the rapid inward sodium current is inactivated.[14]

An important characteristic of such potentials is their slowness of conduction. Together with conduction delay, action potential alternans developed, with alternation of amplitude, duration, and morphology. Fig. 3 shows this phenomenon preceding ventricular fibrillation, together with its association with alternation of ST-segment elevation in epicardial and endocardial leads. In some dogs this progresses to varying degrees of localised conduction blocks, either 2:1 or more irregular patterns, before the appearance of arrhythmias. Arrhythmias were not observed, at least in our pilot studies, in the absence of either preceding electrical alternans, or abnormal potentials, or long conduction delays.

DISCUSSION

The importance of the mechanism of re-entry in the genesis of arrhythmias with respect to the development of spontaneous ventricular automaticity[15] or facilitation by local catecholamine release [16] can be adequately studied only by direct intracellular recording in vivo. Action-potential recording from floating microelectrodes is superior to the monophasic action potential obtained

by induction of tissue injury with a suction electrode.[17] Transmembrane potential recordings and upstroke velocity may not, however, at all times represent true absolute values, because of unavoidable movement artefact and variations in electrode-tip potential, although the time of onset and action potential are exactly reproducible. The early transient prolongation of action potential after occlusion has not previously been reported. This may be akin to the prolonged potentials seen in chronically ischæmic conducting tissue.[18] We have not observed spontaneous ventricular cardiac muscle-cell automaticity in our acute ischæmic preparation, but localised conduction blocks, together with slow conduction and abbreviated action potentials, create the degree of asynchrony necessary for local re-entry. Temporal dispersion of repolarisation may also predispose to re-entrant activity. We propose to make further studies with intracellular recording from two or more ischæmic sites to delineate the extent of such asynchrony in adjacent tissue. We suggest that the relative importance of some ionic and metabolic cellular changes resulting in early arrhythmias may be elucidated with this combination of in-vivo measurements of conduction delay, action potential, and ST-segment current of injury. This technique may also be of value in examining the effect of metabolic or pharmacological interventions.

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IS DEXTRAN 70 A LYMPHOCYTE MITOGEN?

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Summary Dextran 70 ('Lomodex') produced significant increases in thymidine incorporation in a modified whole-blood method of investigating lymphocyte blastogenesis. This blastogenesis occurred at molar concentrations of 7×10^{-6} and 7×10^{-5}. At 7×10^{-7} mol/l dextran alone did not significantly increase thymidine incorporation; however, when this concentration of dextran was added simultaneously with phytohæmagglutinin there was a significant increase in incorporation. Conversely when dextran was added before P.H.A., thymidine incorporation was decreased. These variable effects indicate that dextran 70 may be a B-cell mitogen. Dextran 70 is widely used and these effects on lymphocyte blastogenesis may be clinically relevant.

INTRODUCTION

DEXTRANS are now widely used in clinical medicine as plasma substitutes and as prophylaxis against deep-vein thrombosis. The dextrans used are predominantly linear and are thought to have no important effect on the immune system. However, dextrans with a high degree of branching have been shown to have mitogenic effects on human B lymphocytes.[1]

We investigated the possible mitogenic effect of dextran 70 ('Lomodex') by means of a modified whole-blood method which measures the effect on lymphocyte stimulation.[2] The effect of dextran 70 on the lymphocyte mitogenic response to phytohæmagglutinin (P.H.A.) was also investigated.

METHOD

Defibrinated blood-samples were diluted 1/10 in medium TC199. 200 μl volumes were then distributed into microtitre plate wells. The dose response of dextran 70 was studied by making serial dilutions of the dextran from 7×10^{-8} to 7×10^{-5} mol/l. 16 replicate wells were used for each dilution. The cultures were incubated for 48 h and then labelled for 4 h with triated thymidine (5 μl of 0·5 μCi). Cells and medium were recovered from each well onto filter paper by means of an automatic multiple sample harvester. The filters were added to scintillation fluid and counted for one minute. Two experiments were performed on blood-samples from each of 3 subjects.

The maximum non-stimulatory dose of dextran (7×10^{-7} mol/l) was then used to investigate whether dextran had an effect on P.H.A. stimulation. P.H.A. (Wellcome purified PHA K 2313) at a concentration of 0·1 μg/μl was used. 7 sets of 32 replicate wells were then incubated for 48 h as before but the following were added to the wells for the times indicated: (a) P.H.A. present for the whole 48 h; (b) P.H.A. present during only the last 24 h; (c) dextran and P.H.A. both present for the whole 48 h; (d) dextran for 48 hours but P.H.A. for the last 24 h; (e) P.H.A. for 48 hours but dextran for the last 24 h; (f) dextran present for 48 h; (g) dextran present for the last 24 h. Two experiments were performed on each of 3 subjects.

RESULTS

Significant ($P<0·01$) increases in thymidine incorporation occurred with dextran 70 at molar concentrations of 7×10^{-6} and 7×10^{-5}. At 7×10^{-7} mol/l dextran 70 had no significant stimulating effect (table I). However, stimulation was significantly increased when 7×10^{-7} mol/l dextran was added simultaneously with P.H.A., and

TABLE I—EFFECT OF DEXTRON 70 UPON THYMIDINE INCORPORATION (MEAN±S.E.)

Dextran 70 (mol/l)	d.p.m.	Significance
0	6249±946	
7×10^{-8}	6164±1200	N.S.
7×10^{-7}	6438±756	N.S.
7×10^{-6}	14358±1365	$P<0·01$
7×10^{-5}	23156±971	$P<0·001$

TABLE II—EFFECT OF DEXTRAN 70 ON P.H.A. STIMULATION OF THYMIDINE INCORPORATION (MEAN±S.E.)

Drug	Time (h)	d.p.m.	Significance
None	48	6249±946	..
Dextran 70	48	6438±756	..
Dextran 70	24	6199±1132	..
P.H.A.	48	100 026±5872	..
Dextran 70 P.H.A.	48 48	149 621±2218	P<0·05*
Dextran 70 P.H.A.	24 48	98 076±4980	N.S.*
P.H.A.	24	24 982±2218	..
P.H.A. Dextran 70	24 48	7316±1349	P<0·001†

*Significance compared with P.H.A. at 48 hours.
†Significance compared with P.H.A. at 24 hours.

it was decreased when dextran was added before P.H.A. There was no significant effect when dextran was added 24 hours after P.H.A. (table II).

DISCUSSION

We have demonstrated that predominantly linear dextrans of the type used clinically produce lymphocyte stimulation in vitro. Our findings do not accord with those of others, since predominantly linear dextrans have previously been thought not to produce stimulation.[1] This discrepancy may simply reflect differences in methodology. It has been suggested that phagocytic cells are needed for dextran-induced stimulation;[3] these may have been depleted during the separation procedures used to produce the separated lymphocytes studied by other workers.

The subpopulation of responding cells has not been identified, but both branched dextrans[3] and sulphated dextrans[4] stimulate B lymphocytes. The behaviour of lymphocytes in the presence of both dextran and P.H.A. also suggests that the dextran is a B-cell mitogen, because other non-specific B-cell mitogens show similarly variable effects depending upon the timing of the addition of antigen and dextran.[5]

This complex alteration of immunological responses is probably clinically relevant. Although augmentation of the immune response would be undesirable in many situations—e.g., immune-complex disease—such augmentation could be highly desirable—e.g., during surgery for cancer. Similarly immunodepression would be useful in transplantation.

Our findings are, however, limited both by the number of subjects examined, and by being the results of in-vitro investigations. We hope to amplify our results in clinical studies.

Requests for reprints should be addressed to A.C.B.

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Reviews of Books

Intestinal Ischæmia

ADRIAN MARSTON, F.R.C.S., Middlesex and Royal Northern Hospitals, London. London: Arnold. 1977. Pp. 190. £10.95.

MR Marston's interest in intestinal ischæmia started early in his career, and his subsequent exploration of this subject has been an example of singleness of purpose and scholarship in surgical research. His work on mid-gut ischæmia at the Peter Bent Brigham Hospital was followed by careful observations on ischæmia of the large bowel. He has tidied up many problem areas and he introduced the term "ischæmic colitis." This book is all that is to be expected from such a background. Precise, exhaustive, and written in a lucid but never dogmatic style, it is well illustrated by Peter Drury and by photographs. Two small criticisms: why "acute intestinal failure" for small-bowel ischæmia, whatever its cause may be, and why the apparent lack of urgency in surgical treatment? The condition may be less common than it was some years ago, but a surgeon in a district general hospital may still expect to see one or two cases a year, and a successful outcome depends more on rapid and effective surgery than on painstaking correction of electrolyte and fluid imbalance or on preoperative heparinisation. The author intended to say that rapid surgery is the only possible way to a successful outcome, but the chapter is set out in such a way that this message does not come across clearly. Intestinal ischæmia is as likely to present in an insidious , non-acute form, the outlook then being very much more cheerful. The pathology and management of this form are dealt with in masterly style. The book, though slim and easy to read, is full of references and it should be read by all general surgeons and physicians. The author is to be congratulated: his publishers, who have got five illustrations upside down, are not.

Care of the Elderly

Meeting the Challenge of Dependency. Edited by A. N. EXTON SMITH and J. GRIMLEY EVANS. London: Academic. New York: Grune & Stratton. 1977. Pp. 311. £11·80.

THIS is an important book for policy makers and resource-allocators. The Americans and British have a similar cultural attitude toward the elderly but, largely for historical and political reasons, they have adopted very different ways of dealing with the problems of old age. On both sides of the Atlantic there is mounting anxiety about the management of this dependent section of the community. Britain faces an enormous increase in the numbers of old people who will need health and social services. The function of geriatricians and their relationships with other health disciplines and with the social services have not yet been fully clarified. There are still far too many ancient hospitals, and social services for the elderly are being cut back. Nevertheless, the Americans feel that their position is more critical. Though their elderly burden is not likely to be as heavy as Britain's until the end of this century, systematic study of the aged has been so neglected that there is, for instance, no medical training whatever in geriatrics, no specific medical service for the aged, and hardly any nationally organised community service. In place of these is a huge investment in private nursing-homes which provide the bulk of all hospital beds for the elderly. The U.S. Government's main role here is funding rather than organisation. The Americans know that they are not getting value for money and that the service they are offering will not meet the needs of the elderly in future. It was to take a hard look at these problems that a conference was held in Washington, from which this book is derived. This is a collection of excel-

lent papers by politicians, economists, doctors, administrators, and research-workers, addressed to the following questions—what is the nature and size of the problem of dependency among the elderly; what are the potential and actual resources of health and social services that can be mobilised; what lines of research are likely to be helpful; and what are the implications for public policy and the options available for action? Within this framework there were contributions from over thirty highly qualified speakers. British and American speakers spoke more or less alternately, evincing remarkable contrasts in some cases. The discussion sessions further highlighted these differences, and the keen questions the Americans directed at the British system of care were sometimes difficult to answer. For instance, "Why do geriatricians do so much administration?" and "Is the geriatric department the centre of teaching and information for all caring staff in each area?" There was, however, considerable praise for the British system. There was a remarkable uniformity of agreement on policy. Brain failure is one of the main problems to be tackled in every aspect. It is also clear that nothing should interfere with family willingness to help their elders, but community support programmes should have the highest priorities. There is probably nothing else like this book on the subject of dependence of the elderly.

Children, the Environment and Accidents

Edited by R. H. JACKSON, F.R.C.P., University of Newcastle upon Tyne. London: Pitman Medical. 1977. Pp. 164. £2.95.

FEW people need reminding that accidents are the largest single cause of death in children between the ages of 1 and 15, causing more deaths than the two next commonest causes (congenital abnormality and respiratory disease) combined. A large proportion of hospital admissions in this age-group, as well as an unknown amount of injury treated by general practitioners or without medical help, result from accidents; traffic accidents involving young pedestrians and cyclists take a particularly heavy toll. In common usage the word accident implies that no-one is to blame for what has happened, and that the event is unpredictable and therefore unpreventable. Doctors and others concerned with accidental death and injury in children therefore have a difficult task in putting over the message that accidents can be prevented and that much remains to be done, by legislation and education, to achieve a safer environment for children. The papers which make up this book were given at a conference which brought together those who deal with the results of accidents and those who design the buildings, equipment, and environment which may cause them, and they provide not only an abundance of data (as well as some disturbing illustrations) on the types of accidents and injuries that happen and the age and other characteristics of the children involved, but also raise some of the broader issues which make the subject of accident prevention such a complex one. It is clear that much more research is needed before we can come up with authoritative answers to such questions as why adult supervision appears to have little effect in preventing certain types of accidents; why parents overestimate their children's ability to cope safely with traffic; whether we expect too high a level of safety in our environment and the appliances we use for the price we are prepared to pay (both in terms of money and ease of use, comfort, and æsthetic considerations); why the efforts spent in education on accident prevention appear to be out of proportion to the results achieved; and how we can determine where the line on permitted activities should be set which without hampering a child's initative, yet protects him from hazards he is not equipped to foresee or overcome. The book thus sets out the scope of the problem of accidents in children both as it concerns the professional, be he doctor, designer, or safety official, and the interested member of the public—and child safety is a subject in which few people can afford not to be interested.

Principles of Surgery in the First Six Months of Life

S. FRANK REDO, M.D., Cornell University Medical College, Hagerstown, Maryland, and London: Harper & Row. 1976. Pp. 180. $22.50.

IT is difficult to know for whom this book is intended, although Dr Redo says in his preface that a more definitive and comprehensive discussion and a clearer appreciation of the presentation, diagnosis, and management of the problems encountered in the first six months of life could be accomplished if a book were devoted specifically to them. In the section on hæmangioma, although a large spreading mixed hæmangioma is shown at various stages and its extensive progress is evident, nothing is advised about early treatment when the child was first seen at three weeks; surely active treatment should be undertaken as soon as possible. The excision of a branchial sinus or fistula in the early months of life is better avoided except when there is a very severe infection. In the management of a tracheostomy there is no mention of the injection of saline and its immediate removal by suction. A good deal of space is devoted to abnormalities of the pleura, mediastinum, and lungs, and some of the conditions mentioned are very rare. It is a pleasure to read the advice that inguinal hernias should be treated as soon as possible after diagnosis in the first six months of life, but it is sad to read a recommendation for bilateral exploration even when a hernia has not been seen on the other side. The section on physiology contains an important error—the mean insensible water loss is stated to be 1·0–1·1 ml/kg per 24 h; this should be 1·0–1·1 ml/kg/h. Local analgesia is recommended, even when a pædiatric anæsthetist is available; modern pædiatric anæsthesia offers many advantages to the surgeon. It should seldom be necessary to cut down to set up an intravenous infusion before operation. This book is selective and covers the congenital anomalies and surgical problems in the head, neck, chest, and abdomen during the first six months of life, with a short section on orthopædic problems. The material is similar to that found in other books on pædiatric surgery which cover neonatal problems. There is, perhaps, too much emphasis on problems before, during, and immediately after operation, and it would have been encouraging to have a little more consideration of the long-term problems that result from successful treatment of anomalies in the neonatal period. A specialist pædiatric surgeon has more extensive and specialised texts; for the occasional pædiatric surgeon this book provides a good deal of advice about the immediate approach to a surgical problem but not enough long-term advice for the continuing satisfactory management of the surviving child.

Biomedical Ultrasonics

P. N. T. WELLS, Bristol General Hospital. London and New York: Academic. 1977. Pp. 635. £24, $46.90.

THE importance of medical ultrasound has been increasing and ultrasound now has an established role in a wide variety of clinical circumstances. This book is an impressively comprehensive reference volume written by a physicist for all those working with ultrasound for diagnostic imaging. Though the book is concerned in the main with the biophysical and biological aspects of ultrasound, the basic principles, applications, and instrumentation are all discussed comprehensively. The production of a didactic text on a subject which is rapidly changing is always a problem and sometimes a disappointment. Dr Wells provides an immensely informative, well-illustrated volume taking in most current developments and including a thorough bibliography. All departments concerned with ultrasound should have this book to hand; it will be a standard work of reference which will be of value to medical, technical, and research staff for some time to come.

CHAPTER 5

STREPTOKINASE AND PLASMIN

Any attempt to explore the potential immune effects of streptokinase must allow for the antigenic effect that it is known to have even clinically (Goodman & Gilman, 1975). Fortunately the delay of producing an acceptable assay of fibrinolysis allowed some already out-of-stock streptokinase (kindly given by I.C.I.) to deteriorate further. As I shall show later this had no appreciable fibrinolytic activity but was active antigenically.

The main reason for my interest in this field was the early clinical observations of Thorneset al (1971). Although these were anecdotal there were observed remissions of carcinomata - at least a 50% reduction in tumour bulk. Many drugs used successfully for the treatment of lymphomas have a low remission rate used singly, such as reported for streptokinase. The correct combinations allow much higher remission rates; there seemed such a promise for streptokinase. Obviously a simple immunological role seems likely with such a drug that is antigenic, but other possibilities such as an alteration in the development of the fibrin - stroma about tumours could occur.

Again the work of Thorneset al (1971) suggested a more complex immune effect than that of a simple stimulator of the immune response (such as BCG). Work had also been performed on other proteolytic enzymes such as trypsin. The major problem with any proteolytic enzyme is of course their danger in clincial usage. This was my reason for concentrating on streptokinase, which has been used plentifully. The issue to be decided was the mode of action. Was

it acting as a pure antigen, did it remove blocking substances from either lymphocyte or target cell, or had it another effect?

Streptokinase works by activating plasminogen, this complex is not by itself active at lysing fibrin but can activate plasminogen to plasmin. If there is no plasminogen present then no plasmin can be produced. My method was to divide each experiment into groups; one a control, one using inactive streptokinase (INSK), one using active streptokinase (ASK).

Experiment : To Dilineate "Inactive" Streptokinase (INSK) Activity

a) Antigenicity

10 volunteers suffered intradermal active and inactive streptokinase (100-i.units) with the development of a delayed response (> 12hr) in 8 of 10. The pain and diameter of erythema was not substantially different between the 2 types of SK.

TABLE

Diameter of Erythema evoked by SK (mm).

Subject	Active	Inactive
1	3.5 mm	3.7 mm
2	2.4	2.0
3	2.7	3.1
4	1.5	1.1
5	1.7	2.4
6	2.6	1.3
7	2.0	3
8	4.5	5.1
9	0	0
10	0	0

b) Fibrinolytic Activity

Dose response curves were performed in a P.B.S. solution containing plasminogen (sigma, porcine 1u/ml). The "inactive" SK was substantially less active than the within date SK, there was approximately a thousand fold difference.

TABLE

Effect of the Streptokinases on Fibrinolysis.

	Fibrinolytic Activity n.gms fibrin/ml/hr	
Concentration units/ml	Streptokinase	Out of date
0	10.2 ± 2.9	10.9 ± 8.1
1	126 ± 29	11.7 ± 4.2
10	1717 ± 89	9.1 ± 2.7
100	1512 ± 173	13.2 ± 5.9
1000	17.3 ± 6.2	188 ± 72

Discussion

Within date streptokinase showed the properties expected; it was antigenically active (a delayed response was chosen to obviate a false positive from plasmin induced kinin production), produced enhanced fibrinolysis which decreased above a certain concentration. INSK behaved similarly antigenically but was less active producing fibrinolysis. It was important I felt to check that these effects were similar in the media I would use for the LMT, blastogenesis and in the mouse.

Experiment : Fibrinolytic Activity of Streptokinase in Experiment Systems

The two streptokinase preparations were added in the previous concentrations to Eagles Medium + 10% FCS, Eagles + 10% FCS with washed buffy coat cells, and to Medium 199 with 1 part to 10 of heparinised venous blood. Quintuplicate determinations were made.

Results

Eagles Medium with 10% FCS was similar if not as active, to that of plasminogen; the conventional streptokinase produced fibrinolysis whereas INSK did not. In the presence of cells the results are more complex. The table shows that the inactive drug was active - although not as much as within date SK - in both Eagles Medium with buffy coat cells and Medium 199 with venous blood.

TABLE

Effect of the two types of streptokinase on fibrinolysis derived from Eagles Medium with 10% FCS, with or without buffy coat cells, and from dilute blood in Medium 199.

	Fibrinolytic Activity (ng fibrin/ml/hr)				
Within date SK	0	1	10	100	1000
Eagles Medium with 10% FCS	8.9 ± 3	89 ± 7	151 ± 19	114 ± 19	13 ± 3
With buffy coat cells	17 ± 4	113 ± 11	180 ± 13	177 ± 7	52 ± 5
Medium 199 + 1:10 blood	24 ± 5	149 ± 17	1414 ± 113	715 ± 85	498 ± 47
Mouse venous blood	121 ± 10	198 ± 21	345 ± 31	571 ± 61	212 ± 19

	Fibrinolytic Activity (ng fibrin/ml/hr)				
"Inactive" SK	0	1	10	100	1000
Eagles Medium with 10% FCS	9.2 ± 2	10 ± 3	9 ± 4	17 ± 6	29 ± 12
With buffy coat cells	18 ± 2	21 ± 4	17 ± 3	98 ± 12	114 ± 16
Medium 199 + 1:10 blood	26 ± 3	22 ± 4	27 ± 5	177 ± 21	249 ± 32
Mouse venous blood	122 ± 12	139 ± 8	118 ± 12	214 ± 22	348 ± 32

Discussion

The effect of active SK on FCS seems to be minimal, but this is amplified in the presence of buffy coat cells. This would be expected because of the known production of plasminogen activator (and possibly other fibrinolytically active proteases) from granulocytes and macrophages.

It is difficult to be precise about the difference in response to the inactive SK in inducing fibrinolysis in media with or without cells. Apparent enhancement only occurred in each case at 100 iu/ml, but this ranged from a mere 17 ng/ml/hr in the Eagles Medium to 177 ng/ml/hr in the dilute blood.

In all systems 1 unit/ml of "inactive" SK induced no change in fibrinolysis, but 1 unit/ml of within date SK did produce changes. Peak fibrinolytic activity occurred at 10-100iu/ml - for the within date SK and fibrinolytic activity was diminished at 1000iu/ml. For "inactive" SK, there was no plateau point achieved. As I suggested earlier, streptokinase is active in mouse blood, and behaves appropriately.

The results suggest that these dose response curves could differentiate between fibrinolytic activity and antigenicity, despite mild fibrinolytic activity of the "inactive" SK.

Effect of Fibrinolysis on the LMT

The effect of the two drugs was determined upon the LMT in dose response curves. The LMT was initially examined whole, but without antigen.

(a) Effect on Leucocyte Migration

Migration wells were prepared with leucocyte packed capillary tubes derived from 10 volunteers. Into the wells were placed Medium - with 10% FCS - and the dose response concentration of the two streptokinase preparations. Migration was allowed for the customary time (48hr) when the area migrated was measured. All the volunteers were positive to skin tests with streptokinase.

Results

TABLE

Migrations in presence of streptokinase, skin test positive subjects.

	Migration Area (mm^2)	
Concentration u/ml	Active SK	Inactive SK
0	132 (119-143)	127 (115-143)
1	141 (122-156)	121 (110-141)
10	103 (88-112)	82 (71- 95)
100	98 (85-111)	77 (62- 95)
1000	62 (44- 82)	74 (55- 94)

The dose responses were complex. Migration areas were similar with no drug present; at 1 unit/ml the inactive drug produced no significant effect; the active drug approached a significant effect - but with enhanced migration. ($p < 0.1 > 0.05$). At 10 unit/ml, the inactive drug produced marked and significant ($p < 0.01$) inhibition. This was also seen in the migrations in the presence of active SK. Comparing the two there was a slight but significant difference in favour of the inactive drug ($p < 0.05$). This difference was maintained at 100/iu/ml but not at 1000 u/ml where conversely the active drug produced a lower area although this was not significant.

Discussion

Obviously streptokinase in whatever form will decrease the migration area in most subjects, its effect as an antigen. I think that the cause of the variation in migration with the dose responses was due to changes in fibrinolytic activity and therefore free lymphokine. In the case of the "inactive" SK this would be most marked at the highest dose, while for the active it would be between 10-100 u/ml. In order to test these conclusions, I decided to examine those volunteers I had found that were negative to skin testing with streptokinase, and to examine lymphokine stability in the two stage LMT.

Effect of Streptokinase on LMT in Skin Test Negative Subjects

Two subjects, skin test negative to streptokinase, were bled, and their buffy coat cells used in conventional LMT with the dose response of the two drugs.

Results

TABLE

Migrations in presence of streptokinase, streptokinase skin test negative subjects.

	Migration Area (mm^2)	
Concentration u/ml	Active	Inactive
0	129 (117-143)	126 (112-145)
1	122 (111-140)	131 (119-141)
10	125 (113-133)	129 (117-139)
100	132 (115-151)	123 (111-137)
1000	128 (116-142)	126 (117-138)

There was no significant difference between either drug, or concentration (Mann-Whitney).

Discussion

Inhibition of migration of leucocytes to streptokinase does not inevitably occur and is presumably mediated by its antigenicity, producing lymphokine.

Effect of Streptokinase on the 2 Stage LMT

1. Lymphokine Generation

Lymphocytes from 6 skin test positive subjects were used. They were cultured in supplemented medium containing equal volume dose responses of the two streptokinase preparations, a PBS solution was used in the controls. Following 24hr incubation supernatants were

obtained. The concentration of streptokinase was adjusted to 1000 u/ml in each supernatant which were then corrected for pH. This lymphokine containing supernatant was then tested on lymphocyte depleted leucocytes. Following 4hr migrations the areas were measured.

Results

Inhibition of migration occurred with both types of streptokinase. For the active preparation significant inhibition occurred at 1000 u/ml; the "inactive" produced significant inhibition at 1, 10 and 100 u/ml.

TABLE

Lymphokine generation induced by streptokinase containing medium.

Concentration	Migration Area (mm^2)	
u/ml	Active	Inactive
0	92 (72-100)	93 (74-106)
1	87 (74-96)	65 (36-80)
10	92 (72-108)	62 (48-72)
100	94 (69-116)	64 (40-81)
1000	56 (44-62)	82 (52-106)

Discussion

Lymphokine generation occurred in response to both forms of streptokinase. I presume the cause of the pattern of dose response is from enhanced fibrinolysis and enhanced proteolytic breakdown of L.I.F.

Effect of Streptokinase on Lymphokine Stability

Method

Lymphokine was generated to PPD in the usual manner. It was divided into aliquots and incubated with lymphocyte depleted leucocytes packed into capillaries either with or without the various concentrations of streptokinase. After 24 hours migration areas were measured. Five separate experiments were performed.

TABLE

Effect of Streptokinase on Lymphokine Stability.

	Migration Area (mm^2)	
Concentration uU/ml	Active	Inactive
0	42 (29- 49)	43 (31-52)
1	71 (54- 88)	41 (30-52)
10	82 (62-101)	45 (29-59)
100	84 (61-106)	49 (38-64)
1000	54 (37- 66)	71 (57-92)

Results

The results are shown in the Table. There is a significant increase in area migrated with active streptokinase between 1-100uU/ml which is reduced at 1000uU/ml. "Inactive" only effects a change at 1000uU/ml.

Discussion

These results were compatible with the hypothesis that the lymphokine LIF could be degraded by proteolysis, and that in this model plasmin generated by streptokinase was the cause. To check

that it was not due to a non specific effect, I found the effect of streptokinase on lymphocyte depleted leucocytes in medium alone.

Experiment

Effect of Streptokinase on PMNL Migration (using 6 skin test negative subjects (to SK)).

Method

Six separate experiments were performed; lymphocyte depleted PMNL were prepared in the usual way. To migration wells containing capillaries packed with these cells was added Eagles Medium with varying concentrations of streptokinase.

TABLE

	Migration Area	
Concentration uU/ml	Active	Inactive
1	97 (89-112)	99 (87-114)
10	101 (87-110)	98 (86-114)
100	97 (84-119)	103 (81-121)
1000	81 (69-101)	84 (65-103)
0	100 (88-114)	102 (85-113)

Results

Until the concentration of 1000uU/ml was achieved there was no significant change. At that concentration there was a reduced migration area, which was significant ($p < 0.05$ Mann Whitney) for both types of streptokinase employed.

Discussion

Obviously the effects on the lymphokine can be confirmed up to a concentration of 1000uU/ml. Since both types of streptokinase similarly affected the PMNL at that concentration, I assumed that it was an antigenic effect. It may be mediated from lymphokine release from the depleted lymphocytes (this seems unlikely in view of the lack of response to 1000uU/ml in the mixed LMT). Possibly a selective cytotoxic effect is the explanation.

Conclusion

Streptokinase has two effects on the LMT. First an "antigenic" form, which over the concentrations tested obeyed a logarithmic dose response curve, and secondly an effect due to proteolysis, affecting the amount of free lymphokine.

The next problem was to decide how streptokinase works in the presence of another antigen, for which I chose PPD.

Effect of Streptokinase on PPD Induced Inhibition of LMT

Method

Volunteers and patients were grouped into three Heaf grades; heaf negative, minimally positive and maximal grade (but did not have tuberculosis). Venous blood was taken from each and used to produce buffy coat cells for the LMT. The LMT's were set up with PPD at 150ugm/ml, and the normal varying concentrations of the streptokinases. After 24hr migration the areas were measured by planimetry. All subjects were selected as negative to skin testing with streptokinase.

TABLE

Effect of Streptokinase on PPD Induced Migration Inhibition.

Drug Concentration		PPD	Migration in type of streptokinase (mm^2) Active	Inactive
1. Heaf Negative (n=4)				
		0	124 (106-135)	123 (109-133)
uU/ml	0	150	120 (108-138)	121 (107-131)
	1	150	125 (109-137)	129 (111-140)
	10	150	127 (115-139)	119 (114-135)
	100	150	123 (110-141)	120 (116-137)
	1000	150	124 (109-131)	123 (109-138)
2. Heaf Grade 1 (n=6)				
	0	0	121 (109-136)	123 (108-134)
	0	150	95 (87-114)	97 (89-119)
	1	150	93 (81-119)	95 (88-121)
	10	150	112 (98-133)	98 (85-117)
	100	150	114 (100-129)	93 (90- 96)
	1000	150	91 (80-119)	112 (100-124)
3. Heaf Grade 4 (n=6) patients without tuberculosis				
		0	129 (111-141)	127 (116-138)
	0	150	43 (38- 47)	45 (39- 49)
	1	150	41 (32- 49)	44 (37- 47)
	10	150	52 (43- 63)	41 (33- 52)
	100	150	59 (52- 67)	43 (36- 49)
	1000	150	49 (37- 61)	43 (33- 47)

Results

The table shows the cummulative data, with analysis into each of the three broad heaf groups. The first group, those who were negative to heaf testing, were volunteers who just happened to be negative to both skin testing modalities. The results of the action of PPD showed that there was no migration inhibition. Streptokinase had no effect on the migrations at any concentration.

In the heaf grade 1 group, PPD had an impressive effect, producing significant ($p < 0.05$, Mann Whitney) inhibition. The inactive streptokinase had no effect on this inhibition until 1000uU/ml was achieved. The active drug reduced the inhibition at 10 and 100uU/ml. This was a significant effect ($p < 0.05$, Mann Whitney). The effect was not significant at 1000uU/ml, in fact inhibition was possibly more marked at this concentration, although this did not achieve significance. The inactive streptokinase at 1000uU/ml did, however, produce a significant ($p < 0.05$) change in migration with a lessening of inhibition.

In the final group; patients highly positive on skin testing, and who were subsequently found not to be suffering from tuberculosis, the addition of PPD had a most marked effect ($p < 0.01$) which was partially annulled by active streptokinase at the concentrations 10-100uU/ml ($p < 0.05$) but not by inactive streptokinase. At 1000uU/ml of active SK there was still a significant reduction in migration inhibition ($p < 0.05$).

Discussion

The results show a uniformity of effect which with only minor problems can be explained by the hypothesis that SK can alter lymphokine concentrations by plasmin mediated degradation.

For the heaf negative subjects no lymphokine has been generated and therefore no effect was found by the addition of either PPD or SK. For the heaf positive groups PPD produced the expected inhibition and streptokinase at the concentrations which were fibrinolytically active reduced the inhibition. The only exceptional problem was the effect in group II were 1000uU/ml SK of "inactive" streptokinase did not achieve the expected effect.

One possible problem at such concentrations might be cytotoxicity so I decided that before finding the effect of PPD and SK on heaf and SK positive individuals I would determine the cytotoxic effects of SK at high concentration.

Experiment to Determine the Cytotoxic Effect of Streptokinase and PPD

This was assessed by using trypan blue exclusion performed after cell washing and after the leucocytes had been cultured for 24hr. They were cultured either in Medium alone, or either of the streptokinases at the previous concentrations with PPD 200ugm/ml. Eight experiments were performed upon blood from 4 subjects (all subjects were SK and heaf positive).

TABLE Effect of SK and PPD on Cell Viability

Concentration of Drug		Cell Count	% Non-viable
0		0.79	15.1
Active SK	1	0.81	14.2
	10	0.84	14.7
	100	0.79	14.9
	1000	0.80	15.4

Table Cont/d

Concentration of Drug		Cell Count	% Non-viable
Inactive SK	1	0.80	14.2
	10	0.78	15.1
	100	0.83	14.9
	1000	0.77	14.7

(cell count represents mean x 10^6/ml)

Results

No significant effect was noted either on cell count or percentage non-viability.

Conclusion

Any difference in results would be unlikely to be due to cell death from toxicity.

The Effect of Streptokinase on PPD Induced Migration Inhibition in Subjects Positive to Skin Testing with Streptokinase

Method

Subjects were divided by heaf and clinical status into the following groups : heaf negative but well (n=4); heaf negative but unwell (n=6); heaf mildly positive at grade I, subjects well (n=8); subjects well (n=4) and heaf markedly positive (grade iv) with no evidence of clinical tuberculosis (n=4) and finally those with tuberculosis (n=6). All had had skin tests with streptokinase which were positive to the previous criteria. Venous blood was taken at 9am, the buffy coat cells obtained. These were divided and the LMT's performed for the dose response curve of both streptokinase preparations.

Results

The disease represented by those 'unwell' were varied, but for the heaf negative group was chronic lymphatic leukaemia n=2, carcinomatosis, breast n=1, colon n=1, bronchus n=1 and diverticular disease. For the mildly positive group the diseases were carcinomatosis bronchus n=2, and diabetes mellitus with peripheral vascular disease with gangrene n=2. Since those patients with proven tuberculosis were of Indian origin the heaf markedly positive but well group were of similar extraction but were hospital workers.

I have divided the results into three groups, and they are presented in the following tables. I analysed the results using the Mann Whitney U test.

A. Heaf Negative

The effect in those well heaf negative subjects was as had been found in the earlier experiment. The inactive streptokinase induced a significant inhibition of migration at 10uU-1000uU. There was more marked inhibition at the highest concentration ($p < 0.05$). Those who were unwell had a less marked inhibition to the inactive SK.

Even though there was significant inhibition ($p < 0.01$) since the control and just PPD addition migration areas were similar I felt it justifiable to compare the migration areas at the various streptokinase concentrations. There was no difference at 1uU/ml, significant differences at 10 and 100uU/ml ($p < 0.05$) but not at 1000uU/ml.

TABLE Effect of streptokinase on PPD induced inhibition. Subjects positive to skin testing with streptokinase.

	Migration Areas (mm^2) to									
		Active streptokinase uU/ml					Inactive streptokinase uU/ml			
	Control	0	1	10	100	1000	1	10	100	1000
a. Heaf negative										
Unwell	124 (113-141)	127 (111-144)	135 (110-146)	62 49- 72	67 50- 75	81 65- 98	126 112-136	96 89-113	95 87-113	74 61- 93
Well	122 (110-144)	131 (115-150)	130 (116-142)	112 100-122	105 96-120	54 49- 57	127 110-140	79 65- 97	71 64- 99	67 58- 82
b. Heaf grade 1										
Unwell	120 (108-130)	99 (87-108)	53 47- 59	74 65- 84	76 63- 82	103 91-113	93 87-111	72 64- 80	79 60- 88	65 54- 78
Well	125 (111-141)	84 76- 92	51 43- 60	81 68- 94	88 80-101	60 51- 72	45 36- 51	37 30- 42	42 34- 53	83 74- 95
c. Heaf grade 4										
Tuberculosis	122 (106-140)	58 51- 66	29 26- 33	38 30- 47	62 54- 74	51 46- 64	64 57- 79	56 51- 63	60 51- 67	31 27- 35
Well	124 (107-145)	42 34- 49	31 24-38	59 51- 65	66 52- 81	30 26- 35	29 25- 35	27 25- 31	27 23- 31	44 39- 50

The active drug produced mild inhibition at 100uU/ml ($p < 0.05$) and more marked at 1000uU/ml ($p < 0.01$) when added to leucocytes from fit people. In those unwell at 10 and 100uU/ml there was marked inhibition ($p < 0.01$) which lessened at 1000uU/ml ($p < 0.05$ if control, or 10uU/ml). Again since control migration areas were similar I compared each concentration of streptokinase in the fit and the unwell. 1uU/ml showed no difference. 10uU/ml showed a markedly reduced migration in the unwell compared with well ($p < 0.05$), the same was seen at 100uU/ml. At 1000uU/ml however, the migration was significantly less in the fit ($p < 0.05$)

When the two types of streptokinase are compared the most striking difference is in the low migrations in the presence of the active drug at 10 and 100uU/ml. This difference was significant ($p < 0.05$) unlike the differences at the other concentrations.

Heaf Grade I

Addition of inactive streptokinase to those subjects who were well caused a marked reduction of migration at 1, 10 and 100uU/ml ($p < 0.01$) which was lost at 1000uU/ml ($p > 0.1$). Active streptokinase in the same subjects produced a similarly reduced inhibition at 1uU/ml (p 0.01 if PPD control), but not at 10 or 100uU/ml ($p < 0.01$ cf 1uU/ml). At 100uU/ml, however, there was again significant inhibition.

Comparing the effects of the streptokinase, the differences occurred at 10 and 100uU/ml where the active SK produced less ($p < 0.01$) inhibition than the 'inactive'. And at 100uU/ml the inactive produced significantly ($p < 0.05$) less inhibition.

In the unwell subejcts there was less inhibition to PPD than in the well group ($p < 0.05$). To the inactive streptokinase there was less inhibition than in the 'well' group, at 10 and 100uU/ml ($p < 0.01$) but more at 1000uU/ml ($p < 0.05$). To the active streptokinase there was the same degree of inhibition as in the 'well' group, at 1, 10 and 100uU/ml; less ($p < 0.01$) at 1000uU/ml. Comparing the two types of streptokinase, the differences lie in the migrations achieved at 1 and 1000uU/ml (in the unwell subejcts).

Heaf Grade 4

PPD induced marked inhibition of migration significantly ($p < 0.01$) greater in the 'well' subjects. The addition of "inactive" streptokinase to the wells resulted (at 1,10 and 100uU/ml) in signficantly ($p < 0.05$) greater inhibition which was not seen at 1000uU/ml. Those with TB had less inhibition to 1,10 and 100uU/ml ($p < 0.05$) but more at 1000uU/ml ($p < 0.05$) compared with fit subjects. The active streptokinase produced enhanced inhibition at 1 and 1000uU/ml, lessened inhibition at 10 and 100uU/ml when compared with the control. In each case this was significant ($p < 0.05$ for 1uU/ml of control, 0.01 for the others). The active drug produced enhanced inhibition at 1 and 10uU/ml (p 0.01) producing migration areas similar to those occurring in the 'fit' group. At 100 and 1000uU/ml there was no difference to the PPD control.

So comparing active against inactive streptokinase, the latter was more active at medium concentrations in the group of subjects who were well, and more active at the very high concentrations of 1000uU/ml in those who were ill.

Discussion

A. Heaf Negative

There was an 'antigenic' response to the streptokinase shown best by the 'inactive' streptokinase in the group who were well. This was disrupted in the 'active' streptokinase in the concentrations that produce the most plasmin. In the subjects who were unwell there was less inhibition of migration of streptokinase (inactive). This relative anergy is a frequent accompaniment to the sort of diseases which these patients have if not to the general idea of being ill.

The active streptokinase in the fit subjects produced the expected effect of a lessened degree of inhibiton until 1000uU/ml concentration was achieved; this was not over active fibrinolytically and I presume was similarly not active in degrading LIF.

In the subjects who were unwell the staggering inhibition achieved at 10 and 100uU/ml was surprising. It was similar to the lowest migration area achieved in the fit subjects, occurred at concentrations known to be finrinolytically active and was not seen in more concentrated active streptokinase (1000uU/ml or in 'inactive' streptokinase at these concentrations; none of these were fibrinolytically active. To substantiate the idea that fibrinolysis was important in this inhibition of migration is the finding at 1000uU/ml of 'inactive' streptokinase: in fact the only concentration producing fibrinolysis.

B. Heaf Grade 1

In the fit subjects, an effect similar to synergy occurred when the two cellular antigens were added to the migration wells together. The inhibition provoked by 1uU/ml of inactive streptokinase being quite significant when in the presence of PPD induced inhibition. The expected effects were seen when concentrations of either streptokinase which were fibrinolytically active were used.

In those who were unwell those concentrations of either streptokinase which were fibrinolytically active produced more marked inhibition. The effects of 10 and 100uU/ml of inactive SK did not fit the idea that fibrinolysis was restoring the ability to produce inhibition since the migration areas achieved in inactive streptokinase equalled that of the active drug.

C. Heaf Grade 4

In the fit subjects synergy or addition of effect was again seen. In those with tuberculosis only those concentrations producing marked fibrinolysis provoked inhibition of migration, which when it occurred was as marked as that of the fit subjects.

General Discussion

I think that these results confirm plasmin interferes with migration inhibition, presumably by LIF breakdown. They are suggestive that one mechanism by which generalised anergy can be broken is from fibrinolysis. This would support the idea that fibrinogen or fibrin was acting as blocker substances but the spectrum of plasmin as a protease may include other relevant actions. Is a suppressor substance (a lymphokine) released by suppressor cells; could it be degraded as LIF evidently can be?

Effect of streptokinase on lymphocyte transformation

The analysis of the effect of streptokinase was easier in the transformation of lymphocytes. The reason that neither the antiproteases EACA or a protinin could be used in the LMT to block out the fibrinolytic potential of streptokinase leaving its antigenicity alone was the general effect I had found on inhibition of migration. The analysis of effect would I decided be too complex with so many variables.

In the lymphocyte transformation to PHA, however, I had found that EACA produced decreased transformation but that aprotinin had had little effect. It would be possible, therefore, to partially block the action of the streptokinase induced plasmin. For precision though I decided to complete the experiments first with both types of streptokinase.

Experiment : The effect of streptokinase on lymphocyte transformation

The first essential was to find the degree of transformation likely to be produced by streptokinase. With the relatively short time of incubation used with PHA it was possible that streptokinase had little effect, PPD for instance has only a neglible effect. Unfortunately any possible effect had to be tested carefully because of the possibilities of an effect similar to that formed for Dextran 70.

Method

Streptokinase was added in varying concentrations to 32 wells of dilute blood for each concentration. After 48 hours thymidine was added for a labelling period of 4 hours. The results are the mean of one experiment on each of 4 subjects, all positive to streptokinase on skin testing.

Results

Table - Effect of streptokinase on thymidine incorporation

		Active streptokinase uU/ml				Inactive streptokinase uU/ml			
Dose	0	1	10	100	1000	1	10	100	1000
Mean	8,543	8499	9591	110,451	9114	8399	9335	114,003	7679
SEM ±	829	893	914	1,943	879	941	899	2511	1104

There was a significant effect due to either streptokinase at 100iu/L; there was no significant difference between the two types of streptokinase.

Discussion

Both varieties of streptokinase were active in increasing thymidine incorporation at especially 100iu/ml. There was no significant difference between varieties of streptokinase. This antigenic effect was annoying. To differentiate a complex antigen ligand reaction from a protease ligand reaction would be difficult. I could use plasminogen with a very small dose of streptokinase, commercially acquired plasmin or to find patients without a PHA response.

Obviously using either plasminogen with a small amount of streptokinase or commercially prepared plasmin (via streptokinase plasminogen production) would invoke a further complication. The plasminogen that I could afford was porcine in origin, and could obviously be antigenic.

I therefore decided to test a number of peoples blood to find PHA negative responding people and to find if these would then respond to active streptokinase. I obviously tested the subjects for skin reaction to streptokinase.

Effect of streptokinase on PHA negative subjects

I found two subjects who mounted no lymphocyte response to PHA as tested in the method previously outlined but who were not obviously

TABLE Thymidine Incorporation in response to PHA or Streptokinase; or both

		Patient 1 Mean	Range	Patient 2 Mean	Range
Control		6549	(5218- 8117)	7113	(5821- 9214)
PHA		6698	(4987- 8541)	7087	(5714- 9222)
(Active) SK	1	5998	(4748- 7503)	7049	(5916- 8887)
	10	9219	(6228-13377)	6659	(4833- 8229)
	100	18123	(15144-25022)	19020	(16247-21734)
	1000	8414	(5942-10174)	10121	(7459-13222)
Inactive SK	1	6491	(5001- 8291)	6844	(5621- 9144)
	10	6288	(5177- 8105)	6756	(5716- 9210)
	100	6970	(5511- 8229)	7414	(6100- 9318)
	1000	7174	(5774- 8514)	7776	(6333- 9581)
PHA + Active SK	1	5524	(4466- 7948)	4942	(3842- 5643)
	10	10126	(6411-12279)	8216	(6009-10291)
	100	62277	(58710-67403)	44496	(36942-49090)
	1000	9210	(5884-11444)	7144	(5522- 9410)
+ Inactive SK	1	6337	(5129- 8140)	7149	(5660- 8614)
	10	6511	(5340- 7899)	7008	(5229- 8490)
	100	6994	(5212- 8354)	8226	(4848-11149)
	1000	7241	(5813- 8747)	7956	(3361- 9446)
PHA 48h	1	6214	(4826- 8247)	6648	(4917- 9561)
SK (active) 24h	10	6948	(5323- 9491)	7001	(5544- 8874)
	100	32469	(27510-36555)	21271	(16414-26676)
	1000	7400	(6222- 9101)	10900	(8357-14141)
Inactive	1	6463	(5068- 8214)	9214	(8812-14210)
	10	6411	(5142- 8541)	8699	(6900-13010)
	100	6841	(5521- 9214)	9127	(6141-11496)
	1000	6229	(5100- 7149)	11124	(9241-14741)
SK(active) 48h	1	6988	(5774- 8202)	7295	(5569- 9411)
PHA 24h	10	11798	(8412-14141)	10210	(7741-15150)
	100	7954	(6162- 8714)	7140	(4869- 8212)
	1000	6810	(5211- 8285)	7914	(5216- 7794)
SK(inactive)	1	6564	(4915- 7941)	7994	(6616- 9244)
	10	6337	(5216- 8216)	9125	(8616- 9774)
	100	6514	(4741- 7155)	9520	(5914-11106)
	1000	6984	(4951- 8315	8215	(5213- 9216)

very ill, or malnourished. Both were suffering from carcinoma : one breast, one colonic; neither reacted when skin tested with streptokinase. Neither were on drugs of any sort at the time of testing but both had had resection of their tumour within a week, and had then been subjected to anaesthesia, blood transfusion(s) and analgesia.

I performed the experiment on diluted blood from each subject. Thymidine incorporation was assessed as previously described for control, PHA alone, the streptokinases in the dose responses used previously PHA and the streptokinases added simultaneously at the start of incubation. PHA added first and the streptokinase added 24h later and finally the streptokinases followed 24h later by PHA. 16 wells were used for each test. By these means I had hoped to detect any antigen-ligand interaction.

Results

The results are annotated in the separate table. PHA had no significant effect in either patient; inactive streptokinase was also ineffective. Active streptokinase at 100uU/ml produced significantly enhanced thymidine incorporation in both patients. Minimal enhancement occurred in patient (1) at 10uU/ml and probably (but $p = 0.56$) at 100uU/ml. Patient (2) had enhanced uptake at 100uU/ml.

The combination of PHA and active streptokinase enhanced uptake significantly at 10,100 and 1000uU/ml. This was striking at 100uU/ml, at this concentration the effect was clearly greater than with streptokinase alone : not evident at 10 and 1000uU/ml. PHA and inactive streptokinase did not achieve enhanced uptake.

Analyses to explore a B cell effect using PHA and streptokinase at staggered time intervals showed that active streptokinase, at 100uU/ml effected enhanced uptake of thymidine if added after PHA. If added before, 10uU/ml produced the enhancement. Inactive streptokinase remained inactive except when added after PHA. Especially at 1000uU/ml this appeared to enhance uptake.

Discussion

The most striking liklihood from these results appeared to be that (active) streptokinase was only enhancing PHA uptake when affecting fibrinolysis. This appeared reasonable in that inactive streptokinase did not alter thymidine uptake; and that the dose response curve mirrored that of fibrinolysis.

But it seemed to me that the most exact test of this hypothesis would be if the fibrinolysis could be blunted by Aprotinin which should at the same time blunt the thymidine uptake. On these two patients, therefore, a further experiment was performed.

To test the blunting effect of Aprotinin on streptokinase induced PHA enhanced thymidine uptake

Dilute whole blood was mixed with PHA and with the varying concentrations of (active) streptokinase. To equal volumes was added either aprotinin (to a concentration of 200iu/ml) or an equal volume of isotonic saline. Thymidine incorporation was assessed after 48h incubation with a label of 4h. 36 wells were used in each category.

Table - Effect of Aprotinin on streptokinase induced PHA enhanced thymidine incorporation

	Patient 1	Patient 2
Control	6429 (4916-8214)	7355 (5588-9660)
PHA	6611 (4721-7895)	6985 (5950-9544)
Streptokinase:100uU/ml	22144(16441-26113)	18651(15522-24170)
PHA and SK	66499(62009-7229)	47123(41400-56190)
Aprotinin	6747 (4884-8180)	7572 (6140-9337)
PHA,SK and Aprotinin	28143(24988-31770)	22515(17547-25540)

Results

The data achieved in the control streptokinase and PHA stimulated wells were similar to that seen previously. The combination of PHA and streptokinase again enhanced uptake; more so than either additive on its own ($p < 0.01$). Aprotinin on its own was inactive. In combination it significantly ($p < 0.01$) reduced thymidine uptake, but a difference still remained between it and streptokinase used singly ($p < 0.05$).

Discussion

I suggest that this is further evidence that streptokinase can induce heightened activity with enhanced thymidine uptake, and that this occurs via fibrinolytic activity.

Conclusion

Steptokinase can provoke lymphocyte transformation by (at least) two mechanisms : one by its antigenic nature and one by enhancing fibrinolysis.

The effect of streptokinase induced fibrinolysis in mice

Method

Mice bearing tumour were given either streptokinase (active) or streptokinase (inactive) (SKI) for three days intravenously prior to resection on the fifth day (to decrease bleeding problems more than 24 hours was allowed before operation); and given SK for a further three days after 24h after resection.

Results

There appeared to be no increase in bleeding problem, there was no immediate death in either treatment group. NO anaphylaxis reaction occurred.

Four experiments were performed to see if the number of metastases were altered.

Table - Streptokinase and metastases

Treatment		Mean	Range	Significance
SK.A	n = 7	3	(0 - 6)	$p < 0.05$
SK.I	n = 5	11.75	(6 -17)	
SK.A	n = 9	1	(0 - 3)	$p < 0.05$
SK.I	n = 6	7.5	(2 -13)	
SK.A	n = 6	4.25	(1 - 7)	$p < 0.05$
SK.I	n = 4	13	(9 -18)	
SK.A	n = 8	2.5	(0 - 7)	N.S.D.
SK.I	n = 6	4	(1 - 8)	

This was significantly different in three of the four experiments and the cumulative probability was highly significant. Four further experiments were performed to examine the effect on survival.

Table - Streptokinase and survival

A further 4 experiments were performed to examine survival:

Treatment		1/52	2/52	3/52	4/52	greater than 4/52
SKI	n=10	10	8	6	2	0
SKA	n=11	10	10	10	8	2
SKI	n=12	11	11	5	1	0
SKA	n=12	12	12	11	7	1
SKI	n= 9	8	7	6	1	0
SKA	n= 9	9	9	8	4	2
SKI	n=11	9	7	6	2	0
SKA	n=11	11	10	9	5	1

This was strikingly significant; an occasional mice who had had definite healthy primary tumours resected, survived indefinitely as shown in the last column.

Discussion

Previously experiments using streptokinase have had difficulty resolving its antigenic attributes with the potential to enhance fibrinolysis. By mere good luck I had obtained some outdated streptokinase early in my research - from I.C.I. Limited., which I failed to use. This appeared antigenically intact, but fibrinolytically impaired (see earlier).

I was able to differentiate between these two properties of streptokinase and to show that enhancement of fibrinolysis was active in reducing the frequency of lung metastases and in prolonging life.

In Vitro Immune Response

I had hoped to monitor the immune response in the mouse during the therapy with SK. This was potentially possible by adapting the method used by Luquetti and Janossy in humans (1974), but it became clear in use that the variance was too great. Despite the small volume used mice had to be killed, limiting the value of a method which I had intended would allow simultaneous measurement of fibrinolysis, immune (cellular) response, and antitumour potential.

I have performed three experiments where all the data could be related, the results of which are shown in the table.

Table - Immune response (thymidine uptake to PHA), fibrinolysis, Tumour metastases

8 experiments, mean ± ISD

			PHA		Fibrinolysis		Metastases
1. Control	n=12	n=4	194± 27	n=4	743± 51	n=4	6(2-12)
SK	n=12	n=4	745± 69	n=4	1149±166	n=4	3(0-5)
2. Control	n=16	n=5	266± 22	n=5	729± 11	n=6	7(2-13)
SK	n=16	n=5	844± 81	n=5	1439±196	n=6	1(0-3)
3. Control	n=12	n=4	319± 35	n=4	733± 25	n=4	4(2-9)
SK	n=12	n=4	954± 73	n=4	1388± 77	n=4	2(0-4)
4. Control	n=15	n=5	212± 23	n=5	757± 57	n=5	12(8-19)
SK	n=15	n=5	1414± 98	n=5	1341±171	n=5	4(0-10)
5. Control	n=12	n=4	199± 23	n=4	814± 16	n=4	16(7-21)
SK	n=12	n=4	831± 95	n=4	1412±214	n=4	3(0-5)
6. Control	n=15	n=5	249± 27	n=5	919± 79	n=5	4(0-5)
SK	n=15	n=5	944± 95	n=5	1579±151	n=5	1(0-2)
7. Control	n=12	n=4	213± 27	n=4	624± 27	n=4	21(15-25)
SK	n=12	n=4	744± 84	n=4	1997±195	n=4	7(0-14)
8. Control	n=12	n=4	271± 35	n=4	777± 35	n=4	3(0-5)
SK	n=12	n=4	995±101	n=4	1219±178	n=4	1(0-2)

These results show a clear and significant ($p < 0.02$) improvement in PHA thymidine incorporation, and fibrinolysis with significantly ($p < 0.01$) fewer metastases when the mice were given active streptokinase compared with inactive. With such small numbers in each experiment any attempt at correlation of their three variables was invalid.

Discussion

I have discussed the problems of the Lewis Lung tumour in the methodology - it is unfortunately prone to wide variation in number of metastases. This makes it mandatory to pursue as accurate as possible control of the experiment. Unfortunately, this means that the numbers in each experimental group were limited.

In each experiment there was a trend towards enhanced fibrinolysis, and thymidine uptake to PHA, with reduced numbers of metastases. Although the accepted levels of significance were not achieved in all individual experiments the cumulative probability in each category was highly significant. With such small numbers in each experimental group there was no point in attempting to analyse the degree of correlation between the three variables. Clearly however, they move in the appropriate direction that the hypothesis, fibrinolysis influences the immune system diminishing metastases.

Conclusion

By using two preparations of streptokinase, with different fibrinolytic activities but similar antigenic properties, I was able to show that streptokinase affected lymphocyte function, with enhanced activity; and prolonged mouse survival after resection of the Lewis Lung tumour. I could not disprove the hypothesis that their two effects were linked by enhanced fibrinolysis enhancing the immune system.

CHAPTER 6

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The effect of protease inhibitors on leucocyte migration inhibition to tuberculin extract (P.P.D.)

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Summary. The protease inhibitors, Epsilon Amino Caproic Acid (E.A.C.A.) and Aprotinin were found to significantly increase the inhibition produced by P.P.D. in the Leucocyte Migration Test (L.M.T.). Dose-response curves showed that the most effective concentrations were for E.A.C.A. 0·01 M and Aprotinin 100 I.U./ml.

In a series of 29 subjects with a range of Heaf reactivity this finding was consistent providing that there was a significant inhibition to P.P.D. alone.

At these concentrations, cell viability was unaffected and the effect of the drugs could be reversed by washing.

The exact mode of action of these inhibitors in the L.M.T. requires further elucidation.

INTRODUCTION

Proteolytic agents administered to anergic patients with advanced carcinoma have been shown to restore cell-mediated immune responses. Thornes *et al.* (1974) found that positive tuberculin skin test sensitivity could be demonstrated in previously unresponsive individuals after the administration of Brinase, a protease derived from aspergillus argei. *In vitro* testing of these individuals revealed an increase in E-rosette forming lymphocytes, suggesting an unmasking effect of proteases on T-cells. In addition, incubation of lymphocytes with E.A.C.A. was shown to significantly inhibit E-rosette formation. Hirschhorn, Grossman, Troll & Weissman, (1971) had previously demonstrated that a protease inhibitor, E.A.C.A., was capable of reducing phytohaemagglutinin stimulation of normal lymphocytes. This study was undertaken to investigate the effect of two protease inhibitors, E.A.C.A. and Aprotinin, on cellular immune responses. Initially, the action of these agents has been studied *in vitro* and this paper reports their effect on migration inhibition to P.P.D. in the Leucocyte Migration Test.

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MATERIALS AND METHODS

Antigen

Human tuberculin P.P.D. supplied by the Ministry of Agriculture's Central Veterinary Laboratory was used throughout. The freeze-dried powder (P.P.D. 292) was freshly dissolved in phosphate buffered saline (P.B.S.) on the day of each experiment.

Reagents

E.A.C.A. was obtained from Kabi Vitrum, Aprotinin from Bayer; each were further diluted on the day of the experiment.

Subjects

Informed consent was obtained from 29 people,

21 patients and 8 normal volunteers. Heaf tests were performed, responses measured and individuals graded from 0 to III+ using the criteria established by Pagel, Simmonds, MacDonald & Nassau (1964). The results identified a group of 4 patients with carcinomatosis previously known to react to P.P.D. but now anergic.

The leucocyte migration test (L.M.T.)

This was performed using a modification of the method of Søborg & Bendixen (1967). Twenty ml of preservative-free heparinized blood was mixed with 10 ml of plasmagel (Laboratoire Roger, Bellon) and allowed to sediment at 37° for 20 min. The leucocyte-rich plasma was pipetted off and centrifuged at 250 *g* for 5 min. The supernatant plasma was discarded, the cells washed in P.B.S. at a pH of 7·2, and finally resuspended in Eagles Medium (Wellcome) containing antibiotics and 10% fetal calf serum (Wellcome, lot K1903). Micro-haematocrit capillaries (Hawksley) were filled with the leucocyte suspension and then sealed with plasticine. After centrifuging the capillaries were cut 1 mm below the cell/fluid interface. They were then mounted in ml culture chambers (Sterilin), 2 capillaries to each chamber. At least 4 chambers were used for each experimental group. To each group of chambers was added: medium alone, medium with either drug, medium with P.P.D. (150 or 300 μg/ml), or medium, P.P.D. and either drug, as appropriate for the particular experiment.

After 20 h incubation at 37° the migration areas were drawn using a microscope with a camera lucida attachment. The areas were measured by planimetry and the migration index expressed as the ratio:

$$\frac{\text{migration area in test culture}}{\text{migration area in control culture}}.$$

Dose-response curves

The four subjects were studied with a Heaf 1+ grade, 2 experiments being performed on each subject.

The effects of E.A.C.A. and Aprotinin were then assessed firstly upon their ability to alter migration inhibition on their own, and then upon their ability to change the inhibition to P.P.D., in a dose of 0·1, 0·01, 0·001 M (E.A.C.A.) and 10, 100 and 200 I.U./ml (Aprotinin).

The effect of varying Heaf grade

Migration indices were then obtained for each patient using P.P.D. at 150 μg/ml and testing the effect of addition of either E.A.C.A. 0·01 M or Aprotinin 100 I.U./ml.

Viability

This was assessed by using trypan blue exclusion performed after cell washing and after the leucocytes had been cultured for 24 h with E.A.C.A. 0·1, 0·01 and 0·001 M or Aprotinin 10, 100 and 200 I.U./ml, and in the presence of P.P.D. 300 μg/ml for 1/2 and 24 h. Six experiments were performed upon blood from 3 subjects. Each experimental group consisted of 4 cultures.

Reversibility

Leucocytes were suspended in medium and cultured for 4 h with either Aprotinin at 100 I.U./ml, E.A.C.A. at 0·01 M or in medium alone as controls. The cells were then washed once with P.B.S., resuspended in Eagles Medium and migration tests performed against P.P.D.

Statistics

Significance was evaluated by using the Student's *t*-test and the Mann Whitney *U*-test.

RESULTS

Viability studies

As seen in Table 1, only with E.A.C.A. at 0·1 M concentration was there an appreciable effect of either drug. At this concentration there was a significant decrease in both cell numbers and viability after 24 h culture ($P<0·05$).

E.A.C.A.

At concentrations of 0·001 and 0·01 M, E.A.C.A. was found to have no significant effect on cell migration in the absence of P.P.D. However, at 0·1 M migration was significantly ($P<0·01$) inhibited. In the presence of P.P.D., E.A.C.A. significantly increased ($P<0·01$) the inhibition produced by P.P.D. with an increase in effect from 0·001 to 0·01 M. However, 0·1 M E.A.C.A. failed to produce an increased inhibition. It was decided to explore the action of this drug in exaggerating inhibition, and therefore in further experiments E.A.C.A. was used in a working concentration of

Table 1. Effect of E.A.C.A. and Aprotinin upon total cell counts and cellular viability of human leucocytes

Length of culture	1/2 h		24 h	
Concentration of drug	Cell count	% Non-viable	Cell count	% Non-viable
0	0·82	6·0	0·61	14·3
E.A.C.A.				
0·001 M	0·73	6·2	0·60	14·1
0·01 M	0·74	5·8	0·59	13·9
0·1 M	0·77	6·1	0·49*	16·5*
Aprotinin				
10 I.U./ml	0·79	5·9	0·61	13·8
100 I.U./ml	0·84	6·1	0·58	14·2
200 I.U./ml	0·77	6·3	0·60	14·4

Cell count represents mean of 6 experiments expressing cells × 10^6/ml culture.
* Significance compared with control $P < 0·01$.

0·01 M. These results are illustrated in Fig. 1 showing the means ± 1 s.d. for the 8 experiments at each dose level.

Aprotinin

At all 3 concentrations there was no significant effect on migration in the absence of P.P.D. However, it significantly augmented ($P < 0·01$) the inhibition to P.P.D. being more effective at 100 I.U./ml. The 200 I.U./ml concentration produced less inhibition than expected and therefore 100 I.U./ml was chosen as the working concentration. In Fig. 2 the dose response to Aprotinin is shown for all 8 experiments, values representing means ± 1 s.d.

The effect of E.A.C.A. and Aprotinin in patients of varying Heaf grade

The effects of the 2 drugs were assessed in patients of varying Heaf grade using the working concentrations established in the experiments on cells from Heaf 1+ patients (E.A.C.A. 0·01 M, Aprotinin 100 I.U./ml). The results of these studies are shown in Table 2. Neither Aprotinin nor E.A.C.A. caused inhibition of migration in cells from 6 Heaf 0 patients. In addition, tests were performed in 4 anergic patients with either advanced carcinoma, sarcoid or Crohn's disease who were known to be P.P.D. responsive prior to their illnesses. None of these patients showed inhibition to P.P.D. in the

Table 2. The effect of E.A.C.A. 0·01 M and Aprotinin 100 I.U./ml upon the migration index, employing P.P.D. 150 μg/ml as antigen, in patients of varying Heaf grade

Heaf grade	Number of patients	P.P.D. alone	E.A.C.A. & P.P.D.	Aprotinin & P.P.D.
0	6	0·95 ± 0·125	0·97 ± 0·095	0·92 ± 0·05
0, previously +ve	4	0·98 ± 0·10	1·10 ± 0·125	1·05 ± 0·075
1+	4	0·78 ± 0·085	0·55 ± 0·085*	0·56 ± 0·095*
2+	3	0·76 ± 0·096	0·56 ± 0·05*	0·54 ± 0·120
3+	6	0·61 ± 0·26	0·43 ± 0·21*	0·47 ± 0·16*
Miliary tubercle	6	0·56 ± 0·15	0·35 ± 0·05*	0·30 ± 0·12*

* Significance compared with control $P < 0·01$.

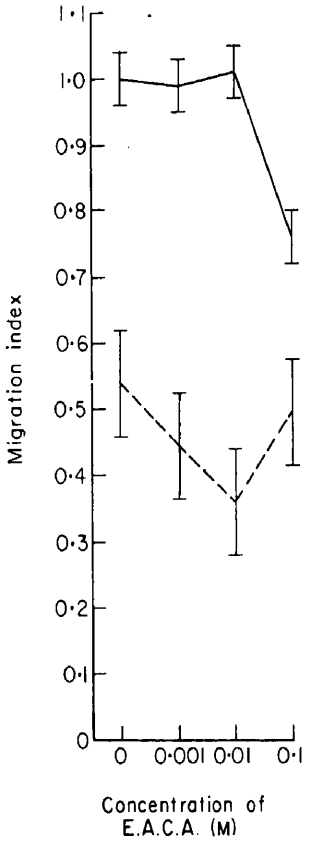

Figure 1. The effect of E.A.C.A. on migration inhibition. Four subjects of Heaf grade 1, two experiments for each subject. Mean ± 1 s.d. (——) Migration into medium + E.A.C.A. alone; (– – –) migration into medium, E.A.C.A. and P.P.D. (300 μg/ml).

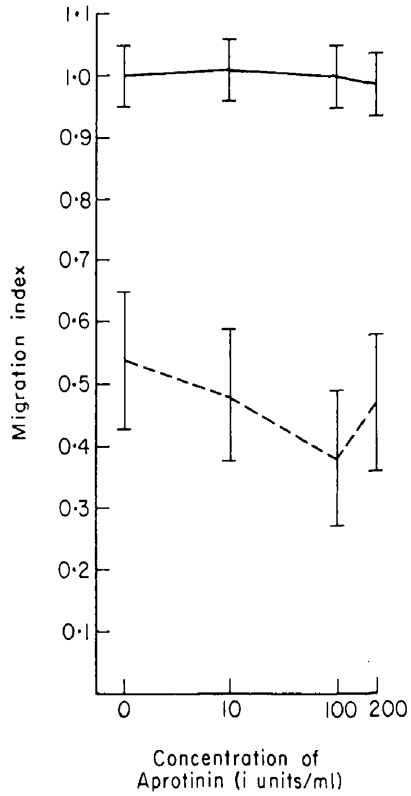

Figure 2. The effect of Aprotinin on migration inhibition. Four subjects of Heaf grade 1, two experiments for each subject. Mean ± 1 s.d. (——) Migration into medium + Aprotinin alone; (– – –) migration into medium, Aprotinin and P.P.D. (300 μg/ml).

L.M.T. and the addition of the drugs caused no significant alteration to the migration index. In the remaining 19 Heaf positive patients, both drugs produced a significant ($P < 0{\cdot}01$) increase in inhibition in each Heaf category.

Reversibility

Four experiments on 2 normal volunteers with Heaf grades of 1+ showed a mean M.I. of 0·75 ± 0·12 for the controls, a mean M.I. of 0·76 ± 0·14 for E.A.C.A. 0·01 M, and a mean M.I. of 0·75 ± 0·13 for Aprotinin 100 I.U./ml.

DISCUSSION

From the work of Hirschhorn *et al.* (1971) and Thornes *et al.* (1974), it had been expected that protease inhibitors would cause a reduction in *in vitro* cell-mediated immune responses. However, this study has shown that two protease inhibitors, E.A.C.A. (0·01 M) and Aprotinin (100 I.U./ml) both increase migration inhibition to P.P.D. in the leucocyte migration test. There were clear dose responses to both drugs and increased inhibition was demonstrated in all Heaf positive grades. Cell viability was unaffected by the drugs at these concentrations and therefore cytotoxicity does not appear to be a significant factor in the results achieved.

In the leucocyte migration test, lymphocytes in the cell suspension produce a leucocyte inhibition factor (L.I.F.) in response to antigen stimulation. L.I.F. acts mainly on macrophages and neutrophils to slow cell migration. The mode of action of the drugs may therefore be:

(1) To stimulate L.I.F. production by lymphocytes.
(2) To reduce L.I.F. breakdown.
(3) To increase the responsiveness of target cells to L.I.F.

If the drugs have a direct action on the lymphocytes

producing L.I.F., positive responses might have been expected in some of the anergic patients, known to be previously tuberculin sensitive. Further, if the drugs were having an 'unblocking' action, then washing cells cultured with E.A.C.A. or Aprotinin would be unlikely to have produced complete reversibility of their effect.

On the results of the present study it is impossible to assess the effect of E.A.C.A. and Aprotinin on increasing the responsiveness of target cells to L.I.F. or on reducing L.I.F. breakdown. Further work is being undertaken to assess the site of action of the drugs in the migration system.

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and Aprotinin (Bayer) 100 iu/ml. The tests were performed with glass microhaematrocrit capillaries (Hawksley) and plastic migration wells (Sterilin).

Cell separation

To produce relatively pure populations of either lymphocytes or polymorphonuclear leucocytes, blood was anticoagulated with preservative free heparin and sedimented with plasmagel (Laboratorie Roger Belon) for 15 min at room temperature. The buffy coat was then removed and carefully layered onto Ficoll/Hypaque. Centrifugation (20 min at 250 *g*) separated lymphocytes from PMNL and the two cell populations could then be harvested. Cells were washed three times with phosphate buffered saline and then resuspended in medium. Stained films of the cell suspensions were examined by light microscopy and if there was more than 5% contamination with the other cell type, the experiment was abandoned.

Lymphokine generation

Lymphokine containing supernatants were generated in an PPD-lymphocyte culture either for 4 or 24 h periods. Lymphocytes, at a concentration of 1×10^5/ml were incubated at 37°, either with PPD or medium alone (for control cultures). At the end of the culture period the cells were spun down and the supernatants obtained. PPD was then added to the control supernatant.

The effect of EACA or Aprotinin upon lymphokine generation. To find the effects of the protease inhibitors on lymphokine generation either EACA or Aprotinin was added to a lymphocyte suspension which was then cultured for only 4h, centrifuged, and the cell free supernatant obtained. Controls were produced by incubating the lymphocyte suspension for the same time in medium and then adding the relevant drug to the cell free supernatant obtained after centrifugation.

Lymphokine stability. The cell free supernatants, either controls or containing lymphokines, could then be used for LIF assay. Although this could be set up immediately, it was also possible to perform an intermediate incubation by keeping aliquots at 37° for either 4 or 24 h before assay. This enabled the stability of LIF in medium containing serum to be determined. The effect of the protease inhibitors on lymphokine stability was studied by adding them to the cell free supernatants before the 4 or 24 h culture period. To obtain the requisite control the drugs were added to the supernatants following either 4 or 24 h culture. LIF assay was then performed.

LIF assay

Fresh PMNL suspended in medium were placed into capillaries the ends of which were sealed with plasticine and centrifuged for 5 min at 150 *g*. The capillaries were cut just below the cell fluid interface and inserted into the wells (one capillary per well) using a minimum of six capillaries in each experimental group. The supernatant to be tested was added to the well. The wells were sealed with glass cover slips and incubated for four hours at 37°. The migration areas were drawn using a microscope with a camera lucida attachment and measured by planimetry.

The effect of either EACA or Aprotinin on LIF assay was determined by adding EACA, Aprotinin, or an equal volume of isotonic saline (for controls) to the cell free supernatants.

RESULTS

The reproducibility of this two stage LMT was investigated by performing three separate assays in six volunteer subjects known to be Heaf positive and with migration indices using the standard LMT and the same concentration of PPD of 0·71 ± SD 0·07. The detailed results are shown in Table 1. There was no significant difference between the migration index achieved in any one subject on each of the occasions they were tested. The mean migration index in the two stage LMT was not significantly different from that obtained for the same subject using the standard LMT.

Table 1. Reproducibility of the two stage method. Three experiments on each of 6 Heaf positive subjects

Subject	Migration index ± SD
1	0·76 ± 0·11
2	0·69 ± 0·07
3	0·54 ± 0·09
4	0·65 ± 0·13
5	0·73 ± 0·12
6	0·74 ± 0·08

Table 2. The effect of intermediate incubation of LIF containing supernatants: two experiments were performed on each of six subjects

Intermediate incubation time (h)	Mean migration index ± 1SD	Significance
0	0·66 ± 0·12	—
4	0·70 ± 0·10	NS
24	0·84 ± 0·09	P <0·01

LIF stability

Twelve experiments, two for each subject, were performed. The results are shown in Table 2; there was a significant loss of activity when lymphokine containing supernatants were subjected to a 24 h incubation before LIF assay. However, there was no significant loss of activity with a 4 h incubation.

Three experiments on each of four subjects were then performed to find the effect of the protease inhibitors on this loss in LIF activity. The results are shown in Table 3 demonstrating that both EACA and Aprotinin reduced the loss in LIF activity.

At 4 h there was no significant difference between the results achieved with supernatants in which a protease inhibitor was added prior to the further incubation compared to supernatants in which the inhibitor was added after the incubation.

These results were important for the structure of the further experiments because using a 4 h culture period clearly avoided any significant effect on the results from loss in LIF activity during the time of the test. Further experiments were needed since the migration indices with protease inhibitor present during the 24 h intermediate culture period, were significantly less than the index achieved by immediate assay with no protease inhibitor.

Table 4. The effect of protease inhibitors on LIF assay; two experiments were performed on each of six subjects

Protease inhibitors	Migration index Mean ± 1SD	Statistical significance
0	0·65 ± 0·11	—
EACA	0·51 ± 0·09	P<0·01
Aprotinin	0·53 ± 0·12	P<0·01

LIF generation

The effect of the protease inhibitors was therefore assessed by using a 4 h culture time. Twelve experiments were performed, three on each of four subjects. Significant inhibition of migration occurred, despite the limited culture time and therefore we assumed LIF was released. Neither EACA or Aprotinin had a significant effect: the mean MI for EACA was 0·84 ± 0·16, EACA control 0·85 ± 0·19; and for Aprotinin 0·80 ± 0·10, Aprotinin control 0·83 ± 0·12. Therefore neither protease inhibitor altered lymphokine generation.

LIF assay

The effect on migration inhibition of adding EACA or Aprotinin to LIF-containing supernatants was assessed in a 4 h assay. The results are shown in Table 4. There was a significant increase in inhibition in the presence of either EACA or Aprotinin ($P<0{\cdot}01$).

DISCUSSION

It appeared logical to divide the conventional LMT into two parts. Our initial experiment (Table 1) showed a reasonable reproducibility and the results

Table 3. The effect of the protease inhibitors on the loss of LIF effect at 24 h intermediate incubation: three experiments were performed on each of four subjects

Protease inhibitors during intermediate incubation	Present: during assay	Mean migration index ± SD —	Significance
0	EACA	0·76 ± 0·07	—
EACA	EACA	0·52 ± 0·06	P<0·01
0	Aprotinin	0·79 ± 0·09	—
Aprotinin	Aprotinin	0·56 ± 0·07	P<0·01

were similar to those obtained in the conventional test.

The second experiment studying the effect of incubation of the protease inhibitors with preformed LIF demonstrated their main action: they prevent loss of activity during prolonged incubation of a LIF containing supernatant. The most plausible explanation of this would be that the serum in the medium contains a proteolytic substance capable of LIF breakdown, which can be inactivated by either inhibitor. This substance may be plasmin, or possibly an activated complement component: EACA is capable of inhibiting the activation of the first component of complement (Soter, Austen, & Gigli, 1975).

Of importance to the further experiments was the finding that incubation of the LIF-containing supernatant for only 4 h produced no significant loss of activity, and it was possible therefore to explore the actions of the proteolytic inhibitors upon lymphokine production, and also their effect on the target cells used in the LIF assay by employing 4 h incubation times.

We found no obvious effect upon LIF generation, which confirms our previous reasoning from the observation that the inhibitors only enhance significant migration inhibition (Burden *et al.*, 1978). There was, however, a marked effect on LIF assay. This was surprising and is difficult to explain, particularly because there was no evidence of a medium effect after only 4 h incubation. A possible explanation is that the inhibitors synergise with LIF in its action on the PMNL target cell. But it may be possible to extend the concept of decreased LIF breakdown. It has been shown that EACA in heparinized whole blood decreases fibrinolysis (Moroz & Gilmore, 1976) by about 65% at a concentration of 10 μmol/ml, although it has little effect upon cell free plasma. The PMNL and contaminants such as platelets may therefore be producing proteolytic enzymes and the inhibition of this activated proteolysis might simply account for the effect of EACA or Aprotinin at this assay stage.

The use of proteolytic inhibitors, of which EACA and Aprotinin are only examples, may increase the sensitivity and selectivity of the Leucocyte Migration Test. The results however must be interpreted with some caution because although we demonstrated no increased LIF production this was only during the short 4 h incubation period, and we are unable to determine if a longer incubation would produce different results.

If the action of EACA and Aprotinin is by decreased proteolytic breakdown of LIF then as both are competitive inhibitors this action may be diminished in the presence of high levels of proteases. Our work used PPD, a relatively pure antigen, but other antigens, especially if derived from tissues, would be likely to augment proteolysis. The concentration of EACA or Aprotinin cannot be increased to counter this because of cytotoxicity (Burden *et al.*, 1978). It may be, therefore, that a non-toxic, non-competitive inhibitor of proteolysis would be more effective.

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EACA AND APROTININ : THE PROTEASE INHIBITORS

Two works stimulated my interest in these protease inhibitors, that of Hirschhorn, Grossman, Troll & Weisman (1971) on lymphocyte transformation; and that of Latner, Longstaff & Turner (1974) on tumour growth. Hirschhorn et al concerned with the early biochemical events of lymphocyte transformation, found that EACA decreased the amount of thymidine incorporation. This study has been criticised because the concentration of EACA was quite high (0.01M) a level close to that which produced toxic symptoms in man. EACA is a synthetic leucine derived amino acid which produces competitive antagonism with the protease acting proponderantly at the site of proteins. It interferes with three proteases : plasmin, lysosomal proteases acting on a variety of substrates - cartilage, protein polysaccharide light and histones; and CI esterase. In vitro fibrinolytic studies (Moroz & Gilmore 1977) have shown that the maximum effective concentration of EACA to reduce spontaneous fibrinolysis is about 0.01M.

The finding of Hirschhorn et al fitted well with the idea of proteases having an important role in the generation of immune response, possibly acting by 'unblocking' lymphocytes. The one difficult previous work to fit into this scheme was that of Latner, Longstaff & Turner (1975) who studied the anti tumour effect of Aprotinin in hamsters and mice. Their rationale sprang from the opposite set of observations upon tumour and host biochemistry. In vivo experiments have demonstrated collagenases and lysosomal enzymes in human tumours (Dresden, Heilman & Schmidt 1972; Shamberger &

Rudolph 1967). In vitro it has been demonstrated that tumour cells can release proteases (Holmberg 1961); proteases can induce proliferation in cell cultures (Burger 1970) whereas protease inhibitors restore cell contact inhibition of movement (Goetz, Weinstein & Roberts 1972). In this context it should be remembered that thrombin is a protease, and several very active proteolytic enzymes eg., Trypsin can induce both fibrin production as well as lysis.

Aprotinin was used by Latner : it is a naturally occurring polypeptide derived from bovine lung marketed as Trasylol by Bayer Pharmaceuticals Ltd. It is a non specific protease affecting plasmin but also trypsin, Kallikrein and thrombin. Latner and co-workers described two separate phenomena; a direct injection of Aprotinin into tumour on either hamster or mouse produced significant tumour necrosis when compared with a control injection of PBS; more convincingly subcutaneous Aprotinin significantly prolonged the survival of mice bearing adeno-carcinomata. They suggested that Aprotinin may prevent the breakdown of lymphokines due to tumour proteases.

With this background I started upon an investigation of the effects of both EACA and Aprotinin upon the inhibiton of leucocyte migration to PPD. I supposed EACA would decrease the inhibition of leucocyte migration; I was unsure of the effects of Aprotinin.

The Effect of EACA and Aprotinin on Leucocyte Migration

The results of this work have been published; Burden, Stacey, Wood & Bell (1978) and I enclose a copy of this paper in the thesis.

Quite clearly both protease inhibitors were active, and enhanced the inhibition of leucocytes to PPD. In the paper we postulated the possible sites of action as either

(i) To stimulate LIF production from lymphocytes (which seemed unlikely since some anergic patients had no positive response).

(ii) To reduce LIF breakdown.

(iii) To increase the responsiveness of target cells to LIF.

This work had both theoretical and practical importance. Theoretically because of the unexpected finding of exaggerated inhibition with EACA, and practically because of the possibility of using either antiprotease to increase the sensitivity and selectibility of the leucocyte migration tests. For these reasons I embarked upon an attempt to demonstrate the mode of action.

Why Did Proteolytic Inhibitors Exaggerate The Response of Leucocyte Migration Inhibition to Antigens?

The question appeared simple to answer by dividing the LMT into two parts; production of LIF and LIF effect. Initially I attempted to use the two stage test employing guinea pig macrophages as the target cells. Lymphocytes from human volunteers are cultured in PPD to increase LIF containing supernatant, control supernatants being produced simultaneously. These supernatants could then be used for the culture fluid into which guinea pig macrophages could migrate. The test was unsatisfactory. The macrophages were obtained by the conventional manner following an intraperitoneal injection of oil, but by the time that the oil had been washed from the cells, the

ability of the macrophage to migrate was grossly impaired. It was possible to produce reproducible results but they depended upon very small areas of migration and, consequently, large errors. Any effect of the protease inhibitors appeared to be masked by this.

I abandoned this approach and adapted the principle to the much more interesting process of dividing the leucocyte migration test into two stages, which I have already described. Using this test it was possible to explore the mode of action of the protease inhibitors at the stage of formation of LIF and upon the effect on the target cell. By incubating LIF containing supernatant it was also possible to explore any possible effect upon the foetal calf serum used in the culture medium. The results are published (Burden, Stacey, Wood & Bell 1979) and I enclose a copy of the paper.

Our results showed that there was no effect on LIF production. The main effects of either Aprotinin or EACA were on lost effectiveness of LIF during incubation in medium which contained FCS and on the target cell. We postulated that this was due to decreased breakdown of LIF : the antiprotease blocking FCS containing proteases and leucocyte proteases.

Further work has confirmed that this is possibly by using supernatants produced after incubation of lymphocytes with PPD in FCS containing medium in the fibrinolytic assay.

Fibrinolysis Due to Leucocytes - Effect of Lymphokines

Method

Supernatants were derived from lymphoycte incubates as described

previously. These were added to lymphocyte depleted polymorphonuclear leucocytes in siliconised glassware. PPD was added to the relevant control supernatant. Following a 4h incubation free fluid was obtained and used in the fibrinolytic assay having been diluted 1 : 1 with Tris NaCl buffer. In a further series of experiments the leucocytes were resuspended and cell containing fluid was used in the assay.

TABLE

Fibrinolytic activity of PMNL and supernatant after exposure of PMNL to LIF containing supernatant. Results of 6 experiments on heaf positive subjects : means ± ISE

	Control Supernatant	LIF containing Supernatant
PMNL free medium	96 ± 29 ng/ml/h	615 ± 125 * ng/ml/h
PMNL containing medium	131 ± 36 ng/ml/h	760 ± 177 * ng/ml/h

* p 0.01 compared with control

Results

There was a low amount of activity in both cell containing and cell free supernatant derived from control cultures, this was significantly greater than that expected from medium alone (p 0.01). However, when PMNL were exposed to LIF containing supernatants there was a marked rise of fibrinolytic activity (see Table).

Discussion

Leucocytes are known to produce fibrinolytic enzymes and this increase in activity due to lymphokine(s) was not unexpected because leucocyte lysozymes and probably plasminogen activator production can be stimulated by the lymphokines.

Effect of EACA and Aprotinin Upon Excess Fibrinolytic Activity of PMNL Exposed to LIF Containing Supernatants

A similar series of experiments were performed to elucidate the effect of the antiproteases.

Method

As previously, PMNL ($1x10^7$/ml) were exposed to control or LIF containing supernatants, but with additional subgroups containing EACA (0.01M) or Aprotinin 200iu/ml. Only cell containing medium was assayed.

TABLE

Fibrinolysis by PMNL, control and activated : Effect of EACA and Aprotinin. Means ± ISE results in ng/ml/hr, 10 experiments one on each of 10 heaf positive subjects.

	Control Supernatant	LIF containing Supernatant
PMNL containing medium	101 ± 29	815 ± 149
+ EACA (0.01M)	88 ± 27	707 ± 118
+ Aprotinin 200iu/ml	81 ± 31	548 ± 69

Results

PMNL containing medium obtained from 10 separate heaf positive subjects gave similar results in both control and LIF containing supernatant to the previous experiment. Either EACA or Aprotinin reduced fibrinolysis in the controls by about 10% (significance $p < 0.05$). When the PMNL were exposed to LIF containing supernatant fibrinolysis was 815 ± 149 ng/ml/h which was significantly reduced by both EACA to 707 ± 118 ng/ml/h $p < 0.01$ (paired t test) and Aprotinin 543 ± 69 ng/ml/hr ($p < 0.01$). Aprotinin reduced fibrinolysis by more than the EACA, and this difference was significant ($p < 0.05$).

Discussion

If our explanation of enhanced leucocyte migration inhibition due to these antiproteases were sufficient, LIF is capable of being activated by PMNL enzymes. This proteolysis can be prevented by EACA and Aprotinin. It would then seem reasonable to expect these antiproteases to exert a similar effect on PMNL fibrinolysis. The reason for the disparity between the EACA and Aprotinin results may not be due to any difference in action in the LMT, but a different effect upon the fibrinolytic system : the less selective antiprotease being more effective.

The hypothesis is only partially supported by these results, one of the antiprotease (presumably EACA) could be more active intracellularly in decreasing the migration.

Conclusion

The attempt to demonstrate an allied effect to the PMNL migration inhibition enhancement, equal for both EACA and Aprotinin failed. The results indicate the virtue of serendipity, for lymphokine(s) containing supernatants enhance PMNL proteolysis.

The Effect of the Antiprotease EACA and Aprotinin Upon Lymphocyte Transformation

Hirschhorn et al demonstrated that EACA reduces spontaneous and PHA activated lymphocyte transformation. This applied to other related synthetic lysine derivatives but not antiproteases with larger molecular weights. Their studies utilised separated lymphocytes; besides demonstrating impaired uptake of thymidine in the presence of EACA they also attempted to define the biochemical site of the lesion.

It is important to confirm their results, since my work on the LMT had suggested an opposite effect. The effect of the antiproteases was tested on both separate lymphocytes and on whole blood.

Methodology

The methods for both the separated lymphocyte and whole blood lymphocyte blastogenesis have been previously described.

Separated Lymphocytes

i) Lymphocytes from two volunteers were obtained on two occasions. The suspensions were divided into aliquots and dose responses for EACA and Aprotinin in control and PHA cultures were performed. The dose

range for EACA was from 0.001M to 0.1M, that of Aprotinin from 1-500 units/ml. Following incubation and thymidine labelling the cells were harvested, washed and the thymidine incorporation assessed (DPM). Tests for cell viability were also performed by trypan blue exclusion with EACA, Aprotinin and PHA.

ii) Thymidine incorporation was assessed in ten subjects lymphocytes in control and PHA cultures exposed to either isotonic saline, isotonic saline 0.1%, EACA 0.01M and Aprotinin 300iu/ml.

iii) I^{125} iodoxyuridine incorporation was assessed in three subjects lymphocytes when exposed similarly.

iv) The effects of antiproteases (EACA, 0.01M, Aprotinin 300iu/ml) on PHA stimulated or control cultures of lymphocytes (from 4 subjects) incorporation of C^{14} leucine was assessed at the various times, 5 mins, 30 mins, 60 mins, 4 hours, 12 hours, 24 hours and C^{14} AIBA at 24 hours.

Results

Antiprotease dose response - the dose response indicated that EACA had no effect on control counts. In the presence of PHA, EACA had little effect at low concentrations, producing maximal effects at 0.1M when there was significant cell killing (see later). Significant depression of thymidine incorporation without cell death with both PHA and EACA occurred at 0.1M. Aprotinin produced slight increase in thymidine incorporation at 100 and 300iu/ml ($p < 0.05$). When 0.1% FCS was added at each dose concentration of Aprotinin, there was no observable effect on increased counts. There was no significant difference in counts with Aprtoinin and PHA at any dose examined.

Trypan blue dye exclusion - the results of experiments on trypan blue dy exclusion have already been published with PPD (Burden et al, 1978). They suggested no significant killing until EACA 0.1M, and Aprotinin at 500iu/ml; PHA and the antiproteases produced a similar effect.

Further confirmation of effect on thymidine incorporation:

TABLE

Effect of Antiprotease upon PHA stimulated thymidine incorporation; means and range (x1000)dpm., ten subjects separated lymphocytes.

Group	Control	PHA
Isotonic saline	6,500 (2- 8)	125,000 (80-144)
Isotonic saline + 0.1% FCS	11,100 (9-15)	115,000 (77-139)
EACA 0.01M	6,100 (2- 7)	39,000 (36- 44)
Aprotinin 300iu/ml	9,200 (7-11)	129,000 (89-146)
Aprotinin 300iu/ml + 0.1% FCS	12,000 (8-15)	132,000 (77-148)

In ten subjects EACA was found to reduce thymidine incorporation in PHA stimulated lymphocytes, from 125,000cpm (mean) to 39,000cpm (mean) - $p < 0.05$ using the Mann Whitney Wilcoxon 'U' test. FCS in a low concentration (0.1%) produced significant increases in counts in the control lymphocytes: $p < 0.05$ (U test) but had no significant effect upon PHA counts. Aprotinin in some patients produced significant stimulation, but this was not as marked as with FCS. When the effect of Aprotinin and 0.1% FCS was examined there was no significant difference from FCS used alone.

I^{125} Idoxyuridine

Rather than thymidine the emitting I^{125} idoxyuridine was added as label, for a shorter label incubation period because of the problem of 'hot' suicide.

TABLE

Incorporation of I^{125} iodoxyuridine into separated lymphocytes effect of EACA and Aprotinin. Means and range (x100)cpm, 3 subjects.

	Control	PHA
Isotonic saline	520 (2- 9)	51,000 (410-630)
+ 0.1% FCS	987 (6-12)	53,000 (430-630)
EACA 0.1M	499 (2- 9)	25,000 (210-320)
Aprotinin 300 iu/ml	875 (7-10)	49,000 (390-610)
+ 0.1% FCS	989 (8-11)	54,000 (440-650)

The results demonstrated a similar trend to that found with thymidine as label : FCS and Aprotinin enhanced counts significantly ($p < 0.05$) in control assays; EACA reduced PHA stimulated counts ($p < 0.01$) but not the controls.

C^{14} Leucine Incorporation

Separated lymphocytes were obtained from 4 subjects, suspended in medium and 10% autologous serum and exposed either to isotonic saline as control or to PHA. Labelling with C^{14} leucine was attempted at various times, either immediately with harvesting at 5, 30 and 60 minutes and 4 and 12 hours, or after 4 hours pre

incubation and harvesting at 24 hours.

The first 3 time intervals - 5, 30 and 60 minutes gave no statistical difference between PHA stimulated and control counts, and neither EACA nor Aprotinin affected the counts (Table).

TABLE

C^{14} Leucine incorporation (dmp), means and ranges of determinations in 4 subjects.

		Control	PHA
	0	125 (65-215)	130 (60-210)
5mins	EACA	127 (63-221)	135 (69-198)
	Aprotinin	119 (57-219)	124 (68-207)
	0	121 (71-191)	125 (75-187)
30mins	EACA	125 (59-201)	131 (77-210)
	Aprotinin	130 (72-210)	135 (73-213)
	0	161 (93-233)	171 (98-238)
60mins	EACA	168 (98-219)	175 (95-235)
	Aprotinin	177 (89-259)	173 (92-236)

At 4 hours there was in one subject a significant increase in C^{14} incorporation - control 145 (89-178) PHA 210 (183-261) dpm ($p < 0.05$, U test) which was inhibited by EACA but not Aprotinin-EACA control 151 (93-183); PHA 185 (152-211); Aprotinin control 141 (85-171); PHA 232 (195-266). The other subjects showed no significant difference between control and PHA stimulated counts, nor with the addition of EACA or Aprotinin.

At 12 hours, 2 of the 4 subjects showed a significant increase in incorporation.

TABLE

Effect of PHA, EACA and Aprotinin on C^{14} Leucine incorporation at 12 hours, 2 subjects, means with ranges expressed as dpm.

	Control	PHA
Control	144 (111-173)	291 (240-349)
EACA	137 (103-169)	209 (171-247)
Aprotinin	159 (123-198)	314 (253-366)

EACA significantly ($p < 0.05$) reduced the PHA stimulated incorporation, but not the control incorporation of C^{14}. Aprotinin had no significant effect.

At 24 hours all 4 subjects showed a significantly increased incorporation of C^{14} Leucine with PHA, enabling the full experiment to be performed.

TABLE

Effect of PHA, EACA, Aprotinin on C^{14} Leucine incorporation (dpm) after 24 hours incubation. Means with range for 4 subjects.

	Control	PHA
Control	210 (169-261)	543 (515-605)
EACA	205 (177-249)	319 (274-376)
Aprotinin	249 (223-299)	589 (544-663)

The variation in results was significantly less after this longer incubation. EACA reduced (significantly, $p < 0.05$) the dpm

in the PHA stimulated group but had no effect on the control counts. Aprotinin in one subject produced significantly increased incorporation in the control analysis (263, range 241-299 dpm compared with 189, 169-234) but in other subjects no significant effect upon either controls or PHA stimulated values.

Uptake of AIBA

This was only assessed at 24 hours. PHA produced significant increases in dpm in all four subjects, and the influence of the antiproteases is shown in the Table. Label period was 12 hours.

TABLE

The effect of EACA and Aprotinin upon PHA stimulation of AIBA incorporation (dpm) means with ranges in 4 subjects.

	Control	PHA
Control	759 (698-825)	1823 (1799-1947)
EACA 0.01M	735 (675-798)	941 (873-1021)
Aprotinin (300iu/ml)	815 (732-878)	1811 (1721-2003)

EACA significantly ($p < 0.05$, U test) inhibited the incorporation of AIBA in the PHA group, having no significant effect in the control group. Aprotinin, significantly enhanced the control counts ($p < 0.05$, U test) but had no significant effect upon PHA stimulated incorporation.

Discussion

I have confirmed the findings of Hirschhorn et al that EACA inhibits the incorporation of thymidine into PHA stimulated lymphocytes.

In a similar analysis to hers, I examined the likely causes. The decreased uptake may have been due to lymphocyte death. There was no morphological evidence for this, and trypan dye exclusion did not suggest that this was important. These were blunt end points, but there was no effect upon control counts, and Hirshchorn et al had shown that the EACA effect could be dispelled by washing - as in my own work (Burden et al, 1978).

The effect of EACA could be detected using the 3 parameters of stimulation, RNA: DNA: and amino acid uptake. The work of Hirschhorn had examined various explanations of this effect - that EACA affected the sodium pump - and that protein synthesis may be diminished prior to RNA or DNA changes. My own data was partly in conflict with their findings. They suggested that C^{14} Leucine incorporation was decreased after RNA synthesis. To test this idea they had examined the effects of EACA or cyclo-heximide an inhibitor of protein synthesis upon Leucine C^{14} incorporation and uridine incorporation which marked RNA synthesis. Cycloheximide inhibited protein incorporation but had no effect upon uridine incorporation. I do not believe that the exact time of the effect of EACA is important, most of the substrates examined are transport dependent and this may be the ultimate explanation rather than Hirschhorn's postulate that EACA interferes with proteolytic control of DNA transcription sites. Even if the latter explanation holds, the clinical features are still relevant, and it was in this context that further experiments were performed on whole blood cultures.

Effect of EACA on PHA Transformation in Whole Blood Thymidine Incorporation

Method

The whole blood method was used, diluted whole blood was delivered in 200uls aliquots into a multicell culture container, and to 24 replicates equal volumes of either isotonic saline, or EACA in 0.0001, 0.001, 0.01M concentration was added. At each concentration of EACA, PHA was added to 12 replicates. The experiments were repeated on 12 subjects.

Results

The results are expressed in the Table. At no concentration of EACA was there a significant change in control counts. PHA stimulated values fell exponentially from 156,067 ± 19,094 (SEM) to 23,126 ± 2,406 at the concentration of 0.01M, obviously highly significant ($p < 0.01$).

Effect of Washing

Diluted whole blood from 4 subjects was divided into 2 aliquots, isotonic saline or PHA was added to either. Each was then divided into a further 2 aliquots, isotonic saline or EACA was added. After 24 hours incubation the four aliquots were centrifuged (5 mins, 10,000rpm) the supernatant discarded, cells resuspended in medium and recentriguged (x3). The controls - not containing PHA - were then each divided into a further 2 aliquots. Isotonic saline or PHA was added.

TABLE Effect of EACA or Aprotinin on Lymphocyte Transformation and the action on PHA Enhanced Thymidine Uptake (c.p.m.; means ± SEM)

	PHA added	
0	156,067 ± 19,094	1,974 ± 194
EACA 0.0001M	131,199 ± 20,137	1,888 ± 207
0.001M	78,897 ± 8,976	1,915 ± 315
0.01M	23,126 ± 2,406	2,027 ± 199
Aprotinin		
100 iu/ml	162,071 ± 21,123	10,175 ± 1210
200 iu/ml	155,052 ± 18,798	12,716 ± 1354
300 iu/ml	160,001 ± 20,054	15,566 ± 1419

The 6 groups were then plated out in the conventional manner; but thymidine was added immediately and the cells were harvested after 12 hours.

Results

As can be seen in the Table, there was an effect due to EACA during the incubation with the whole blood containing PHA. This was apparently reversed by washing, because the cells exposed just to EACA, then washed and exposed to PHA responded as well as those not exposed to EACA.

TABLE Effect of washing upon EACA effect

Addition	Isotonic Saline	Saline/PHA or EACA washed	PHA alone
Isotonic saline	9,543 ± 921	33,129 ± 2098	121,111 ± 20,987
EACA	8,981 ± 954	35,292 ± 1897'	206,214 ± 14,143*

' not significant compared with control

* compared with control $p < 0.05$

Effect of Aprotinin upon PHA Transformation in Whole Blood

Method

Using the whole blood method, 24 replicates of 200uls aliquots of dilute whole blood was delivered to a multiple cell culture plate, and equal volumes of either isotonic saline, Aprotinin in 100, 200, 300iu/ml was added. PHA was added to 12 replicates of each

concentration. The experiments were repeated on 6 subjects.

Results

Aprotinin provoked a significantly ($p < 0.05$) increased incorporation of thymidine at each concentration examined, showing a dose response (Table). In the presence of PHA this increase was not statistically significant in any experiment.

Discussion

The effect of EACA on a whole blood system is analagous to that on separated lymphocytes; the finding that the effect can be eliminated by washing suggests a reversible change. Since Aprotinin does not have this effect, it would seem unlikely that the effect occurred at the level of the cell membrane. The explanation for the enhanced thymidine incorporation produced by Aprotinin perhaps lies in its possible antigenicity. It is derived from Bovine lung, anaphylactic effects have been reported clinically (Com. Safety of Medicines) even though rarely. An alternative explanation could lie in its complex antiprotease behaviour. It is an efficient antagonist of protease acting on the Kinin system. It would be entirely logical to predict that the kinins would be capable of inducing or enhancing lymphocyte transformation. They are known to be chemotactic, and probably do induce lymphokine release. A multihit system whereby kinins stimulate the production of inflammation encouraging the migration of lymphocytes, and simultaneously produce early lymphocyte transformation might be expected.

It seemed reasonable to extend this work in vivo. The Lewis Lung sarcoma model was chosen. The method and the problems associated

with it have already been explained. The first problem was to define the effect of subcutaneous EACA in fibrinolysis in C57 black mice.

The Effect of EACA upon Fibrinolysis in Mice

I could find no published data on EACA effects in mice. I therefore conducted experiments to find if it affected fibrinolysis, its effective or biological half life, and to find if the concentration which produced inhibition of fibrinolysis was toxic. The initial experiments were performed in vitro.

In Vitro Fibrinolysis

Method

Blood anticoagulated with EDTA from 20 C57 black mice was pooled. An EACA dose response was conducted, to four aliquots was added either isotonic saline or 0.0001M, 0.001M, 0.01M EACA. The blood was then used in the fibrinolytic assay as outlined previously.

Results

Although EACA at the lowest concentration tested had little effect, the reduction of fibrinolysis was significant ($p < 0.05$, unpaired test) and it appeared that 0.01M was approaching the plateau level (Table). This would be predicted if spontaneous fibrinolysis in the mouse was only partially plasmin dependent. EACA had no significant effect upon the buffer controls.

TABLE

In vitro effect of EACA, results of pooled blood of 5 experiments, results expressed in ng/ml/hr, means ± SE

	Tris Na Cl.	Blood
Isotonic Saline	15 ± 1	743 ± 47
0.0001M	16.± 2	710 ± 59
0.001M	14 ± 1	678 ± 35
0.01M	15 ± 2	664 ± 61

In Vivo Fibrinolysis

Mice were randomly chosen from a batch of 40, to either enter a control group (10 animals) or to receive various quantities of EACA subcutaneously; 100uls, 200uls, 500uls, or 1ml. (10 animals in each group, concentration of EACA was 1.4G/L). Four hours later the animals were exanguinated, blood anticoagulated with EDTA, stored on ice (for less than 4 hours) and assayed for fibrinolysis.

Results

No animal suffered adversely from the injections of EACA. The table shows the results of the experiment.

TABLE

	Fibrinolysis (ng/ml/hr)	Means (range)
Isotonic Saline	721	(689 - 779)
100uls EACA	710	(681 - 752)
200uls EACA	694	(655 - 731)
500uls EACA	654	(619 - 688)
1ml EACA	644	(596 - 674)

The results were analysed using the Mann Whitney U test. There was no significant effect of 100uls of EACA, but at 200uls there was slight inhibition, which became significant at 500uls, $p < 0.05$. The difference between 500uls and 1ml of EACA was not significant.

The Effect of Time on EACA Inhibition of Fibrinolysis

Mice were chosen randomly and assigned to isotonic saline as control or EACA (500uls) as previously described. Following the administration of EACA or isotonic saline they were culled at 24 hours and blood obtained for the assay.

Results

Fibrinolysis in the control group of 10 mice was similar to that obtained in the last experiment : 713 ± 63 ng/ml/hr but was statistically different from that given EACA, 621 ± 44 ng/ml/hr, $p < 0.05$.

Discussion

In the mouse EACA effects inhibition of spontaneous fibrinolysis, both in vitro and in vivo. The quantity needed for a maximum degree of inhibition was at least 200uls subcutaneously, and as 500uls produced significant inhibition of fibrinolysis 24 hours later, this would allow some accumulation if given daily.

The Effect of EACA on Growth and Body Weight

Any deleterious effect that EACA may have on mice bearing tumour may be mediated by the vague but important area of malnutrition. EACA,

500uls sc, was therefore given to 10 animals daily for two weeks from when they were three weeks old. They were compared with a peer group (n=10) who were given 500uls injection s.c. of isotonic saline, and a further peer group of 10 mice given no injections. There was no apparent difference in body weight between the groups. The most significant effects was a localised loss of hair about the EACA injection sites.

Conclusion

EACA causes no discernable toxic effect to mice when given as 500uls subcutaneously each day; at this dose it significantly reduces spontaneous fibrinolysis.

The Effect of EACA upon the Lewis Lung Tumour

Method

The mice were prepared as described above; for one week before tumour resection half of each group were given EACA. They were chosen at random. The controls were given isotonic saline, adjusted to comparable pH with infused CO_2.

Results

Initial experiments examined the frequency of metastases and as can be seen in the table there was a significant increase in the number of metastases in the EACA treated group.

	mean number of Mets (range)		Significance (U test)
1. Control n=9	12.5	(7 -19)	$p < 0.05$
EACA n=9	17.5	(14-27)	
2. Control n=10	4	(0 -11)	$p < 0.01$
EACA n=11	18	(16-32)	
3. Control n=12	7.5	(1 -10)	$p < 0.05$
EACA n=13	10	(7 -15)	
4. Control n=8	9	(6 -13)	$p < 0.05$
EACA n=7	12.25	(9 -17)	

Life Span

A comparison was made between survival of EACA or isotonic saline treated mice.

Post Resection Survival

		Number alive at:-		
		1 week	2 weeks	3 weeks
EACA	n=6	6	5	1
Control	n=7	7	6	2
EACA	n=11	10	8	0
Control	n=10	10	9	3
EACA	n=12	12	10	2
Control	n=13	13	12	5
EACA	n=9	8	6	0
Control	n=8	8	8	3

The simplest method of analysing this data appears to be a rank test which would be significant ($p < 0.05$).

Discussion

Both the frequency of metastases and the survival post resection were adversly affected by EACA, at a dose which impaired fibrinolysis. This does not necessarily imply however, that alteration of fibrinolysis was the predominant cause.

Effect of Aprotinin upon Lewis Lung Metastases

Latner et al, (1975) described two separate phenomena : a direct injection of Aprotinin into tumour in either hamster or mouse produced significant tumour necrosis when compared with an isotonic saline control injection; subcutaneous Aprotinin significantly prolonged the survival of adenocarcinomata bearing mice.

This work clearly required repeating but with the more reasonable control of foetal calf serum injections.

Effect of Aprotinin on Mouse Lung Metastases

Method

As previously described, but Aprotinin 500ul or F.C.S. (500ul) given subcutaneously bd for 10 days before resection and then 1ml bd of either material for 10 days.

Results

Four experiments were performed, the results are catalogued in the table.

TABLE

Effect of Aprotinin upon number of metastases means (range) of 4 experiments.

	Means (ranges)	Significance
Aprotinin n=4	4 (0- 7)	
Control n=4	4.25 (3-12)	N.S.D.
Aprotinin n=6	8.25 (4-16)	
Control n=6	11.5 (8-17)	$p < 0.05$
Aprotinin n=5	8.75 (4-12)	
Control n=5	8.25 (5-19)	N.S.D.
Aprotinin n=7	2.25 (0- 4)	
Control n=7	8.75 (6-16)	$p < 0.01$

Discussion

The results appear unequivocal, despite using the more stringent control of F.C.S. Aprotinin significantly reduced the frequency of metastases in two of the experiments, and the cumulative probability was less than 0.05. Experiments were then performed to examine the survival of Aprotinin treated mice.

Effect of Aprotinin on Mouse Survival

Method

As previously described.

Results

Post Resection Survival	1 week	2 weeks	3 weeks
Aprotinin n=9	9	9	8
Control n=8	7	7	4
Aprotinin n=7	6	6	5
Control n=7	7	4	2
Aprotinin n=6	6	5	4
Control n=6	5	4	1
Aprotinin n=9	9	8	8
Control n=10	10	9	4

In each experiment the Aprotinin treated mice fared better than the controls, the overall significance being less than 0.01.

Discussion

The hypothesis that manipulation of fibrinolysis could alter the immune response, and have an effect upon tumour growth, or metastases must have been disproved by these results, but only if this quantity of Aprotinin does affect fibrinolysis.

Effect of Aprotinin on Fibrinolysis in Mice

(i) In Vitro Effects

Aprotinin dose responses were performed by using pooled blood from C57 black mice, Aprotinin 1, 10, 100, 200 units were added to each ml of blood.

Results

TABLE

Effects of Aprotinin on fibrinolysis.

	Mean
Control	754 ± 55
Aprotinin 1	743 ± 49
10	748 ± 57
100	731 ± 35
200	692 ± 29

The control of fibrinolysis, using in this case isotonic saline as control was 754 ng/ml/hr. Only at 200iu/ml was there any significant reduction in fibrinolysis to 692 ng/ml/hr ($p < 0.05$). This may be achieved during treatment with Aprotinin.

(ii) In Vivo Effects

Mice were randomly entered into a series of groups: control injection for 10 days or Aprotinin s.c. for 10 days. Mice were exsanguinated at 1h, 3h and 6h after the injection; fibrinolysis was then measured.

Results

			Mean ± Range	Significance
Isotonic Saline		n=11	735 (687-783)	
Aprotinin	1h	n=11	699 (651-731	
	3h	n=11	729 (671-753)	$p < 0.05$
	6h	n=11	738 (695-754)	

The mean fibrinolysis of the control group, 735 ng/ml/hr was significantly different from those mice killed one hour after an injection of Aprotinin, 699 ng/ml/hr. There was no difference at 3 or 6 hours.

Discussion

This is at the end of the series of injections, which could have produced an accumulative effect. This would be unlikely with these results showing only an alteration of fibrinolysis over an hour.

The inhibition of fibrinolysis was therefore minimal during the course of injections, although effects on other proteases may be more prolonged; Aprotinin being a relatively non specific antiprotease.

Even this minimal inhibition of fibrinolysis should, according to the hypothesis which I have been examining, produce an impaired immune response. This should then increase primary tumour growth and frequency of metastases. This sequence is untenable without modification. Either a continual change in fibrinolysis is important, or the alteration in the immunity was not harmful. Alternative conceptions are equally attractive; for example, that the kinin - or lymphokine breakdown is more important.

Conclusion

I have shown that both antiproteases; the specific EACA and the more general Aprotinin; have marked effects upon the immune system.

Both enhance leucocyte migration inhibition to the antigen PPD. This only occurs when inhibition already occurs. The site of action appears to be compatible with the hypothesis that lymphokine(s) produced in response to the antigen could be lysed by proteases; this lysis could be reduced by appropriate antiproteases. It would be difficult to extrapolate these results to a clinical situation. The control balance within the migration chamber between protease and antiprotease would be considerably different from in vivo. The lymphokine concentration in a migration chamber would be greater than in vivo, except perhaps in circumstances such as abscess formation, or around necrotic areas of tumours.

EACA inhibited lymphocyte transformation; Aprotinin occasionally produced transformation, not surprising in view of the foreign nature of the product. These results support Hirschhorn's contention that EACA acts at an intracellular level. Extra cellular depression of fibrinolysis achieved no depression of lymphocyte depression. This would not disturb the hypothesis that fibrinolysis alters the immune response, for the effect on receptor metabolism, or particularly receptor antagonist formation may take time which these experiments do not allow, before the presentation of the ligand. The number of PHA receptors (if they occur as definite places) may be considerably greater than the number required to be bound with PHA before transformation occurs. Receptor blockade under these circumstances would have little effect. The final 'extenuating circumstance' is that fibrin may be the blocker; but in the presence of heparin fibrin will not be produced.

In the animal model EACA acted as predicted. Aprotinin had a minimal effect of fibrinolysis and a converse effect on metastases. This may imply a more marked contribution from the kinin system to metastases formation than presently imagined.

CHAPTER 7

CONCLUSION

This study commenced with the complex picture of the influence of clotting, and fibrinolysis on the spread of carcinoma. There was only a minor hint that the clotting and fibrinolytic systems interfered with immunity. It would be too generous to expect that a series of enzyme cascade systems controlling coagulation would control the immune system, but I believe that I have presented evidence to suggest a considerable involvement. Reasons to account for this involvement would be pure speculation but there may be important pathophysiological principles highlighted by them.

My choice of methods was, of course, partly limited by what was at the time possible. Both immunological methods that I chose had their problems, which have been discussed elsewhere. Most objections are those applied to any in vitro test. The most valid objection to the study is that I did not include any examination of macrophage activity. The best answer to the objection that in vitro tests are an invalid picture of the actual, is to show repeated correlation between the test result and a clinical event : thus massive salt intake relates well with hypernatraemia. I have attempted to demonstrate this for both the LMT and lymphocyte transformation.

The animal experiments were an attempt to test hypotheses developed in the in vitro tests. I had hoped to continue the experiments further in clinical experiments. These I did not perform for two reasons; time and the inability to show a clear

trend towards depressed fibrinolysis in patients with carcinoma that I had expected. Although the study would have been more complete with a controlled trial, this would take at least five years to complete and had already been commenced by others (Bowel Tumour Cooperative Study).

The main thread of the study has been shown in the chapters on streptokinase and on fibrinogen. If streptokinase has an immunological effect, one would expect fibrin (ogen) degradation products to have a similar effect producing a positive feedback. The negative, or damping effect would be that of fibrin deposition. Inflammation produces this and presumably the balance between fibrin deposition, fibrinolysis with splitting to fibrin degradation products has importance in localised inflammation.

The whole concept of fibrinolysis enhancing various aspects of immunity was challenged by the initial findings that EACA and aprotinin increased the inhibition to antigen in the leucocyte migration test. Although the explanation that the antiproteases were decreasing the lymphokine breakdown seemed likely, it was difficult to construct a satisfactory test of the hypothesis. The two stage method which I finally used seemed to provide good evidence, and I think provides further ideas about the effects of fibrinolysis in localised inflammation.

I doubt that the action of EACA in the transformation of lymphocytes to PHA is illustrative of any particular physiological action of the antiproteases since the effect appears to be largely intracellular. Its clinical use may be underplayed, its effects may be apparent on lymphocyte function before any untoward effect on plasmin.

Aprotinin has two problems, it is minimally antigenic, and has mainly an activity against kinins. Its actions in the animal model was surprising. It can either be accepted as disproving the idea that fibrinolysis affects the immune response, or, more reasonably that since aprotinin at the dosage used had such a little effect on fibrinolytic activity, its effects were from alteration of other protease systems, for example the kinins.

I would suggest, therefore, that I have produced evidence that streptokinase induced fibrinolysis can enhance lymphocytic responses, streptokinase induced fibrin degradation products can enhance lymphocyte responses whereas fibrinogen depresses immune activity. In localised inflammatory conditions a further problem may occur : lymphokines may be inactivated by a protease (which itself can be inactivated by EACA or aprotinin); in these conditions then fibrinolysis impairs immune-mediated responses.

While heparin may have a physiological or pathophysiological role, the other anticoagulants which I examined are clearly of pharmacological interest. Warfarin is known to be mildly cytotoxic, as I discussed earlier, and behaved as such, interfering with the in vitro immune tests. In the mouse the known effect of warfarin reducing the number of viable metastases held, and since the animals survived, this effect was clearly more important than its action as immune depressant. Dextran, and heparin I found to be a B cell stimulant. The exact nature of which B cells were stimulated I could not evaluate.

I had finally hoped to be able to describe which patients might best be aided by altered fibrinolytic activity. Unfortunately when I studied patients about to undergo operation (Reilly and Burden, 1980) although patients with carcinoma had a lower fibrinolytic activity pre-op, some rebounded post-op to high levels. To describe the appropriate patient would need standardising the time of sampling. I had wanted to sample blood draining from tumours, but this was inpractical for most tumours. I also attempted to define the fibrinolytic activity of tumours. These gave impressively variable results, presumably due to which area (necrotic, tumour, vascular stroma,) was needled. The variability was too great to allow comparison.

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