INFLAMMATORY AND OTHER FACTORS PRODUCED WHEN SENSITISED LYMPHOCYTES ARE STIMULATED WITH ANTIGEN

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ABSTRACT

The tuberculin reaction is thought to be a specific immune response by sensitised lymphocytes to the antigen. It is characterised histologically by infiltration with mononuclear leukocytes.

In experiments described in this thesis, mononuclear leukocytes from peripheral blood of tuberculin-sensitive guinea-pigs, and consisting of greater than 90% small lymphocytes, were stimulated in vitro in serum-free medium with the tuberculin antigen, PPD, for 3 days. The concentrated cell-free supernatants from these cultures produced an inflammatory reaction when injected intradermally into unimmunized guinea-pigs. Supernatants from cultures without antigen did not produce an inflammatory response. The reaction was characterised by erythema and induration reaching a peak at 4-6 hours with perivascular infiltration of polymorphonuclear and mononuclear leukocytes. The supernatants possessing inflammatory activity contained a factor inhibiting the migration of peritoneal macrophages from unimmunized guinea-pigs.

In experiments in man, it was demonstrated that peripheral blood leukocytes, consisting of 10% lymphocytes and 90% polymorphs from tuberculin-sensitive donors, were inhibited in their movements by the presence of PPD. This inhibition, which did not occur in cells from tuberculin-negative donors, was blocked by 1 uM actinomycin D. In supernatants from PPD-stimulated serum-containing cultures of mononuclear cells from individuals sensitive to tuberculin, there occurred a factor capable of inhibiting the movement of polymorphs. This factor was not produced by cells from tuberculin-negative donors and was not demonstrable in the absence of serum. However, serum-free cultures of peripheral

blood mononuclear cells from tuberculin-positive donors when stimulated with PPD released a soluble factor producing erythema and induration when injected intradermally into unimmunized guinea-pigs. This reaction, characterized by an infiltration of mononuclear cells and polymorphs, was maximal at 4-6 hours. There appeared to be a correlation between antigen-specific release of this inflammatory factor and the ability of supernatants to enhance migration of polymorphs.

EDWARD JENNER (1749-1823)
From a portrait by J. R. Smith, 1801

FIGURE 1. Edward Jenner

CASE IV.

MARY BARGE, of Woodford, in this parifh, was inoculated with variolous matter in the year 1791. An efflorefcence of a palifh red colour foon appeared about the parts where the matter was inferted, and fpread itfelf rather extenfively, but died away in a few days without producing any variolous fymptoms*. She has fince been repeatedly employed as a nurfe to Small-pox patients, without experiencing any ill confequences. This woman had the Cow Pox when fhe lived in the fervice of a Farmer in this parifh thirty-one years before.

* It is remarkable that variolous matter, when the fyftem is difpofed to reject it, fhould excite inflammation on the part to which it is applied more fpeedily than when it produces the Small Pox. Indeed it becomes almoft a criterion by which we can determine whether the infection will be received or not. It feems as if a change, which endures through life, had been produced in the action, or difpofition to action, in the veffels of the fkin; and it is remarkable too, that whether this change has been effected by the Small Pox, or the Cow Pox, that the difpofition to fudden cuticular inflammation is the fame on the application of variolous matter.

CASE.

FIGURE 2. A page from Jenner's "An enquiry into the Causes and Effects of the Variolae Vaccinae" (1798) describing a delayed hypersensitivity reaction to the smallpox with Jenner's comments upon the reaction.

When the antigen Tuberculin is injected into the skin of an animal or man sensitised to that antigen by previous contact with the tubercle bacillus, a delayed hypersensitivity reaction to the tuberculin may occur. This is characterised by the gradual development of a dull red raised area that is firm and indurated, appearing after a few hours but not reaching its maximum size until 24 or 48 hours. Subsequently the reaction fades over the next few days and completely disappears. The elucidation of the means by which this reaction occurs comprises the scope of this thesis.

INTRODUCTION

Probably the first description of a delayed hypersensitivity reaction appeared in 1798 in Edward Jenner's "An enquiry into the Causes and Effects of the Variolae Vaccinae". Alone, it is worthy of mention, but in addition, Jenner's interpretation of the phenomenon must be considered remarkable (Figures 1 & 2).

Here the suggestion is made of an enduring change that occurs in the body after exposure to infection, enabling it to reject in an accelerated manner, matter to which it has become sensitised. Here also that change is linked to the inflammatory response of the vessels within the skin and attention is drawn to the usefulness of the reaction as a diagnostic test for immunity.

Jenner thus demonstrated insights into the nature of the immune process anticipating even some modern ideas concerning its nature. Indeed, it was not until 1890 that a similar phenomenon was described by Robert Koch (Figure 3) in his investigations into tuberculosis.

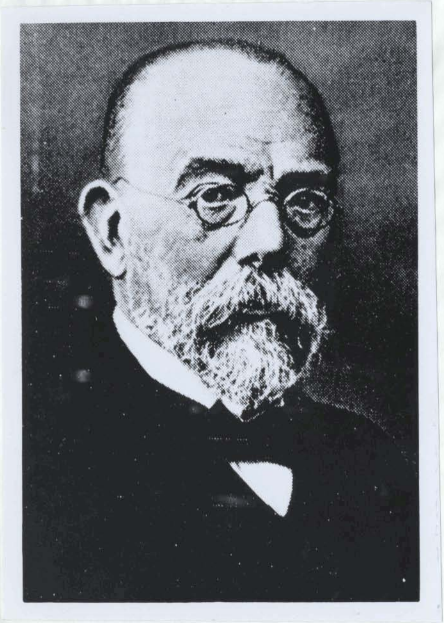


FIGURE 3. Robert Koch

"...If a healthy guinea-pig be inoculated with a pure cultivation of tubercle bacilli, the inoculation wound generally becomes sealed, and seems to heal up during the next few days. It is only in the course of from ten to fourteen days that a hard nodule is formed, which soon opens, forming an ulcerating spot which persists until the death of the animal; but the case is very different if an already tuberculous animal be inoculated. The most suitable animals for this experiment are those that have already been successfully inoculated four to six weeks previously. In the case of such an animal also the small inoculation wound becomes sealed at first, but no nodule is formed, a peculiar change taking place at the point of inoculation. Already, on the first or second day, the spot becomes hard and dark-coloured; and this is not confined to the point of inoculation, but spreads around to a diameter of 0.5 to 1 centimetre. During the next few days it becomes more and more clear that the epidermis thus changed is necrotic. Finally it is thrown off, and a flat ulcerated surface remains, which generally heals quickly and completely, without carrying infection to the neighbouring lymphatic glands. Thus the inoculated tubercle bacilli act quite differently on the skin of a healthy guinea-pig and on that of a tuberculous one. But this remarkable action does not belong exclusively to living tubercle bacilli, but also in the same degree to dead ones, whether killed by low temperatures of long duration, which I first tried, or by boiling heat, or by certain chemicals."

Subsequently, this reaction became known as the Koch phenomenon. Koch himself followed this work up with the development of a bacterial extract with the same activity, which he called Old Tuberculin (Koch, 1891). This consisted of a glycerin broth culture of human-type, Mycobacteria tuberculosis, grown for 6-8 weeks and then concentrated by boiling, finally being freed from bacilli by filtration.

Koch also described the systemic tuberculin reaction, which may follow the administration of tuberculin to sensitive individuals by systemic, subcutaneous or even intradermal routes. Its features depend upon the dose administered and the degree of sensitivity, in its mildest form being characterised by fever, flushing and general malaise and when most severe producing hypotension, hypothermia and weakness which may result in death.

The cutaneous tuberculin reaction was further investigated by Epstein (1891) and von Pirquet (1907) using the contact technique which bears his name, and by Mantoux (1910) who introduced the now-standard technique of intracutaneous testing. The conjunctival reaction test was also introduced at this time (Calmette, 1907). These reactions were all employed in the diagnosis of tuberculosis.

It was von Pirquet and Schick (1903) who first recognized the tuberculin reaction as an allergic phenomenon analogous to other recently described immunological reactions such as anaphylaxis or the Arthus Skin Reaction.

However, in 1921, Zinsser showed that tuberculin sensitivity differed from other recognized forms of allergy in that it could not be readily transferred passively using serum. Furthermore, in 1925 Zinsser and Mueller suggested that "these substances upon which (bacterial) allergy depends may possess protective functions different from and based on a different mechanism from those possessed by antibodies".

Indeed, as was later pointed out both in animal experiments and clinical experience, there appears to be a close parallel between host resistance and the prevailing level of tuberculin sensitivity (Wilson et al., 1940; Heimbeck, 1936, 1949; Daniels et al., 1948; Bloch, 1955).

Certain evidence subsequently appeared to demonstrate a dissociation between tuberculin sensitivity and immunity (Wilson et al., 1940; Raffel, 1950; Rich, 1951; Crowle, 1958). However, as Mackaness (1967) has pointed out, in a critique of this evidence, one cannot imply, from the absence of skin reactivity, that immunological reactivity at the cellular level is absent. The interrelationship of immunological response and antigen sensitivity, which may determine actual resistance to disease, makes the analysis of immune mechanisms extremely difficult.

The histological characteristics of tuberculin and anaphylactic type of skin reactions were clearly distinguished by Dienes and Mallory (1932, 1936). Whereas in those reactions mediated by antibody, there was a rapidly developing oedema of the skin quickly followed by an intense polymorphonuclear leukocyte (PML) infiltration reaching its height in 1-6 hr; in the earliest and mildest tuberculin reactions, the cellular infiltration was at all times almost purely mononuclear. In their experiments, reactions began at 4 to 6 hr and were maximal at 24-48 hr. Early on lymphocytes were seen within small vessels, notably venules; later they were seen in perivenular cuffs often several cells deep. Oedema was never striking, leading to the conclusion that cellular accumulations must be responsible for a significant part of the induration palpable at the tuberculin reaction sites. Polymorphonuclear leukocyte infiltration was seen only when there was necrosis.

In agreement, Laporte (1934) in his studies of the tuberculin reaction was able to correlate the degree of polymorphonuclear response with the severity of the response; in particular with the extent of the necrosis. The cellular exudate was found to consist of monocytes with frequent lymphocytes and, in severe reactions, up to 20% eosinophils.

Gell and Hinde (1951) and Gell (1958) also essentially confirmed the previous findings, in addition noting the initial infiltration to consist of monocytes and histiocytes with a few lymphocytes and a small but definite proportion of granulocytes.

Wesslen (1952) also investigated the histology of the reaction in tuberculous rabbits. He found that after 3 hr considerable infiltration was spread over the dermis, polymorphs being interspersed with lymphocytes. Only after 24 hr did mononuclear cell types predominate.

In 1963, Boughton and Spector re-examined the histology of the tuberculin reaction in the guinea-pig. They found that in the first 2-3 hr there occurred a small or moderate inflammatory infiltration of predominantly polymorphonuclear leukocytes from the vessels. By 5-6 hr, polymorphonuclears were no longer seen in the perivascular areas, where mononuclears now predominated. They were, however, visible in the intervascular spaces, into which they had apparently moved. At 8 hr the emigration of leukocytes, predominantly polymorphonuclears from the vessels began again, the area being heavily infiltrated with granulocytes as well as being oedematous. But by 24 hr emigration of cells was subsiding, polymorphonuclears were moving away from vessels into the tissues and perivascular cell collections were dominated by mononuclear cells that had left the circulation with the granulocytes but which had become immobilized around the vessels. These authors considered the tuberculin reaction to have a biphasic pattern, with a minor peak at 3-4 hr (probably a non-specific reaction to tuberculin) and a major peak at 8-12 hr, due to specific sensitisation.

The passive transfer of delayed hypersensitivity experimentally by Landsteiner and Chase in 1941 confirmed the importance of mononuclear cells in the tuberculin reaction, and Chase (1945) was able to transfer tuberculin-sensitivity to normal guinea-pigs using peritoneal exudate cells from sensitised animals. Such cell suspensions consist primarily of macrophages and lymphocytes. In these experiments, skin reactions became positive following intravenous administration of the cells, within 20-36 hr.

Subsequently, passive transfer of tuberculin sensitivity with pure lymphocytes was demonstrated by Kourilsky et al. (1952) and Wesslen (1952).

But if the dependence of the tuberculin reaction upon specifically sensitised cells, probably lymphocytes, was thus demonstrated, the means by which these cells produced the inflammatory response was still unsettled.

In 1932 Rich and Lewis had shown that the cells that would normally migrate out from explanted fragments of spleen or bone marrow in the presence of tuberculin, were inhibited and killed when the explants were from tuberculin-sensitised animals. These cells were tentatively identified as a mixture of monocytes or macrophages and polymorphonuclear leukocytes. Thus the tuberculin reaction could consist of an immunologically specific necrosis of sensitive cells with a secondary inflammatory response.

A number of papers were published confirming Rich and Lewis' original findings (Heilman & Seibert, 1946; Favour, 1947; Fabrizio, 1952; Waksman, 1953; Gangarosa et al., 1955) whilst others were not able to confirm them (Baldridge & Kligman, 1951; Marks & James, 1953).

Waksman and Matoltsy (1958) in agreement with the latter group of authors were able to demonstrate a superior cell survival of macrophages from sensitised guines-pigs exposed to tuberculin. Even more important was the apparent proliferation of intermediate mononuclear cells in these cultures. There appeared to the authors to be a morphological progression from small lymphocytes to these cells, which were peroxidase negative, but took up neutral red stain and had a scanty basophilic cytoplasm filled with dark granules or vacuoles. Mitoses were not seen in the cultures but the findings quite definitely argued against a purely destructive role for tuberculin in tissue culture.

There followed the demonstration by Carstairs (1961, 1962) that the small lymphocyte from human peripheral blood can transform morphologically under the influence of a phytohaemagglutinin extracted from the red kidney bean, Phaseolus vulgaris. The cells become large, strongly basophilic and blast-like with prominent nucleoli and subsequently divide. These findings were confirmed by Marshall and Roberts (1963), Schrek and Rabinowitz (1963) and Lindahl-Kiessling et al. (1963).

This led Pearmain et al. (1963) to examine the effect of tuberculin upon lymphocytes from tuberculin-sensitive individuals in tissue culture. They found a similar morphological transformation resulting in cell division, whereas cells from tuberculin negative donors did not divide. These observations suggested that an immunologically specific recognition of antigen was made by lymphocytes of sensitised individuals in vitro. This phenomenon was also reported by other workers (Cowling et al., 1963; Schrek, 1963; Marshall & Roberts, 1963).

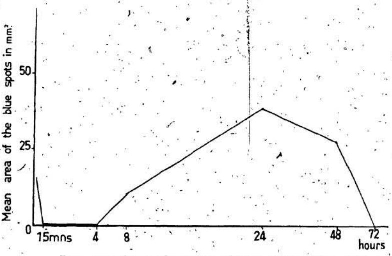
Thus lymphocytes of a sensitised individual or animal are stimulated by the presence of specific antigen to become large active protein synthesizing blast cells (Torreli et al., 1966; Asofsky & Oppenheim, 1966) and it therefore becomes possible that the tuberculin reaction itself might be linked to some product of the lymphocyte. These cells, specifically sensitised to tuberculin and capable of movement through many tissues of the body (Gowans, 1959; Marchesi & Gowans, 1964; Gowans, 1966) might respond to the presence of that antigen with the production of substances eliciting an inflammatory response, of which the cutaneous tuberculin reaction is but one manifestation.

Others had considered this possibility at an earlier period. Zinsser and Tamiya (1926), Bălteanu et al. (1938), Carrere and Quatrefuges (1952) had attempted to show, by the incubation of sensitised cells with antigen in vitro, the presence of substances which would cause a reaction of the tuberculin type upon intradermal injection into normal animals. However, the results of these experiments were never convincing.

Similarly, Waksman and Matoltsy (1958) had incubated sensitised peritoneal exudate cells and lymph node cells with antigen for up to 24 hr and then injected the resuspended cells in culture fluid into the skin of normal animals without producing an inflammatory response except upon one occasion. They used concentrated cell suspensions in saline and plasma and suspected that most of the cells were damaged or killed by the end of the incubation period.

However, in 1959 Johanovsky reported that, after incubating cells from rabbits sensitised to BCG with dilute tuberculin, the cell-free supernatants provoked an inflammatory response when injected intradermally into normal animals. The inflammation appeared within 24 hr and control supernatants did not produce such a reaction. He tested peripheral blood cells, spleen cells and cells from cisterna chyli.

These results were repeated in 1960 by Johanovsky, using sensitised guinea-pig peritoneal exudate cells cultured with tuberculin or diphtheria toxoid at a cell concentration of 1.25 - 7.5 x 10^7 cells/ml for periods of from 2 to 20 hr. Again the supernatant fluids were injected intracutaneously and the reactions measured at 24 and 48 hr. Lymphocyte cultures from animals sensitive to tuberculin more frequently produced supernatants capable of eliciting an inflammatory response,



Increase of vascular permeability at the sites of intradermal injections of old tuberculin (200 μg.) or purified tuberculin I.P.48 (20 units) in BCG-vaccinated guinea pigs. Variation in relation to the delay of injection.

N.B. At time 0, 15 min., and 4 hours, the calculations were made by subtracting the corresponding areas of the blue spots in normal controls.

FIGURE 4. (From Voisin and Toulet, 1960).

when incubated with that antigen, than when incubated with diphtheria toxoid as a control. However, the specificity of the response was not always demonstrable. The difference between the control and experimental groups was said to have been most marked when those animals with the most intense and those with negative reactions were compared.

In 1960, Voisin and Toullet, investigating the modifications of capillary permeability in immunological reactions using Evans blue dye, had been able to demonstrate a delayed increase in permeability beginning at 4-8 hr after the intradermal injection of tuberculin into sensitised guinea-pigs. The change in permeability reached its peak at 24 hr (see Figure 4).

A possible mediator of the change was the lymph node permeability factor (LNPF) described by Willoughby et al. (1963) in extracts of lymph node cells from tuberculin-sensitive guinea-pigs. This material, which was distinguished from histamine, serotonin, bradykinin, kallikrein and globulin permeability factor by pharmacological means, produced a significant increase in vascular permeability after intradermal injection into normal animals. There was an associated emigration of leukocytes beginning with an early infiltration of polymorphonuclear leukocytes and followed within 6 hr by a cellular response consisting of almost entirely mononuclear cells. These cells persisted for several days (Boughton & Spector, 1963).

Extracts of the skin-bearing tuberculin reactions demonstrated a rise in concentration of LNPF in parallel with the development of the lesion, waning as the reaction subsided. However, normal as well as sensitised lymph node cells were found to contain the factor LNPF and both released it into the medium upon incubation with PPD. In addition, a

TABLE A.

Skin Reactive Factors

Type of Cell	Cell Concentration	Time in Culture (h)	Stimulant and Concentration	Concentration of Supernatant	Peak of *in vivo* reaction (h)	Nature of Infiltrate	Authors
GUINEA-PIG							
Sens.LNC	$1.2x10^7$	24	PPD (25ug/ml)	10x	8-12	mono. (4 h)	Bennett and Bloom (1968)
Sens.LNC, PEL	$1x10^7$	22	BGG (0.2-2mg/ml)	1x	6-10	mono. + poly. (6 h)	Dumonde et al. (1968, 1969)
Sens.LNC, PEL	$1x10^7$ (PEL) $5x10^7$ (LNC)	24	PPD ($10ug/10^7$ cells)	1x	3	mono. + poly. (6 h)	Pick et al. (1969)
Sens.LNC	$3.5x10^6$	24	PPD (25ug/ml)	10x	4-16	?	Heise and Weiser (1969)
RABBIT							
Sens.LNC	$5x10^7-1x10^8$	6	PPD (100ug/ml)	1x	24	mono. + poly. (12 h)	Krejci et al. (1969)

LNC = Lymph node cells

PEL = Peritoneal exudate lymphocytes

mono. = mononuclear cell

poly. = polymorphonuclear cell

preparation of minced rat liver incubated with rat serum had similar properties upon intradermal injection to LNPF (Spector & Willoughby, 1964).

Thus, this material may not be immunospecific but simply be some substance extractable from lymph node cells and other living tissues. LNPF is said not to be destroyed by proteolytic enzymes and to have a molecular weight >100,000 (Schild & Willoughby, 1967) and these authors have raised the possibility that the active principle of LNPF may be ribonucleic acid (RNA) or one of its degradation products. RNA was shown to have inflammatory properties similar to LNPF. The presence of LNPF at the site of a tuberculin reaction may thus be the result of cell infiltration and not of specific release of a mediator of the response.

A number of authors have recently described soluble factors derived from lymphocyte cultures and able to cause skin inflammation. The essential features of these are summarized in Table A.

In the experiments of Bennett and Bloom (1968) the intradermal injection of the concentrated supernatants from stimulated cultures caused skin reactions which appeared in 3-5 hr, were maximal at 8-12 hr, and disappeared by 30 hr. These reactions consisted of moderate induration and erythema with a diameter of 11.8 ± 0.63 mm whilst control supernatants produced smaller erythematous reactions of 6.9 ± 0.98 mm and no induration. Their experiments were carried out in inbred Strain XIII guinea-pigs to rule out possible reactions due to foreign histoincompatibility antigens.

Histological examination of the reactions showed, at four hours, an exudate consisting nearly entirely of mononuclear cells. Later, neutrophils appeared so that by 14-16 hr an exudate of approximately

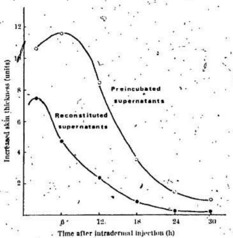


FIGURE 5. The time course of increased skin thickness following intradermal injection of 0.1 ml volumes of peritoneal lymphocyte culture supernatants. Sensitised lymphocytes obtained from guinea-pigs immunized with BGG in Freund's complete adjuvant. Antigen in culture: BGG (200 ug/ml). 0-0, Pre-incubated supernatants, ●-● reconstituted supernatants (Dumonde et al., 1969).

equal numbers of mononuclear cells and neutrophils was present in the dermis and subcutaneous tissue.

Dumonde et al. (1969) used ^{125}I-labelled human serum albumin to assess early changes in permeability after injection of the cell-free supernatants from guinea-pig lymphocyte cultures. They found that stimulated supernatants produced approximately three times the accumulation of radioactive label that control supernatants did. Much of this accumulation occurred within the first hour. A more delayed component was shown by a measurement of the induration produced (see Figure 5). This revealed a differential increase in skin thickness between 6 and 10 hr after intradermal injection, at which time there was a cellular infiltration of the skin test sites with mononuclear and polymorphonuclear cells. It was noted that lymphocyte sonicates did not produce such an inflammatory response, suggesting that acute cell death was not responsible for the effect.

The supernatants examined by Krejci et al. (1969) for inflammatory properties, were derived from rabbit lymph node cell cultures and were tested by intradermal injection in normal guinea-pigs. Changes in vascular permeability were assessed by the use of Evans blue dye. Control supernatants from cultures incubated without antigen produced an immediate erythema with an increase in vascular permeability maximal at 30 minutes and subsequently fading. A slight inflammatory infiltration of polymorphonuclear leukocytes occurred. In contrast, supernatants from stimulated cultures initially produced an area of considerable pallor, peaking at 30 min and subsequently fading. There was no change in vascular permeability at this time. A small area of central erythema then occurred, gradually enlarging, with an associated increase in permeability,

FIGURE 6. The histological picture of the inflammatory reaction 24 hours after the intradermal injection of the supernatants from a culture of rabbit lymph node lymphocytes stimulated with PPD 100 ug/ml. H & E stain, x 150 (Krejci et al., 1969).

replacing the pallor in 4-5 hr. About 12 hr later the erythema was followed by induration and at 24 hr there was a considerable reaction with the macroscopic appearance of a delayed hypersensitivity reaction. The histological findings at 12 and 24 hr showed an inflammatory infiltration of polymorphonuclear and mononuclear cells (see Figure 6).

The results of the experiments of Heise and Weiser (1969) demonstrated that supernatants from PPD-stimulated sensitised lymphocytes elicited skin reactions, whereas supernatants from cultures of normal lymphocytes incubated with or without antigen were much less active, as were supernatants from sensitised lymphocytes cultured without PPD. Of some interest was the non-specific inflammatory activity of supernatants derived from sensitive or normal macrophages incubated with PPD (see Figure 7). Reactions in all cases appeared at about 4 hr and began to fade gradually after 16 hr, disappearing by 48-72 hr. The histology of these reactions was not examined.

Pick et al. (1969) investigated in considerable detail the Skin Reactive Factor (SRF) released by antigen-stimulated lymphocytes. In their hands the injection of supernatants from the sensitised lymph node cells and peritoneal exudate lymphocytes incubated with PPD produced an inflammatory reaction with erythema and induration reaching its peak in 3-6 hr. There was said to be no such activity in supernatants from non-sensitive lymphocyte cultures incubated with antigen. However, these results were not given. Although there appears to be a definite difference between control and stimulated cultures, here, as in previous papers, the supposedly unstimulated supernatants also produced at times rather marked inflammatory responses. Material from unfractionated peritoneal exudates and purified peritoneal macrophage cultures also produced inflammatory

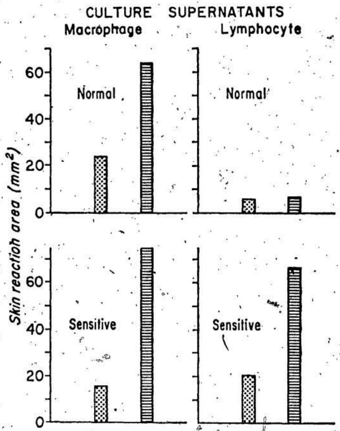


FIGURE 7. The skin reactions (mm^2) in normal guinea-pigs elicited with supernatants from purified tuberculin-sensitive lymphocytes and macrophages cultured in the presence of PPD. Dotted histograms indicate supernatants to which PPD was added after the culture period. Cross-hatched histograms indicate supernatants from cultures incubated with the antigen. (Heise and Weiser, 1969).

reactions but here the differences between stimulated and control supernatants were much less striking.

Microscopically at 6 hr there occurred a mixed polymorphonuclear-mononuclear leukocyte infiltration in the deeper parts of the dermis, the cells being mainly perivascular. There were either equal numbers of both cell types or a predominance of polymorphonuclear leukocytes. At 24 hr the infiltration was reduced but similar in composition (see Figure 8).

The production of inflammatory supernatants was prevented by the presence of actinomycin or puromycin, the latter reducing protein synthesis to less than 5% of previous levels. In addition, pepsin destroyed the inflammatory activity of the supernatants whilst ribonuclease or deoxribonuclease had no effect. Fractionation of the supernatants on Sephadex G-200 produced a peak containing most of the inflammatory activity with a molecular weight approximately that of serum albumin.

A number of other substances are detectable in the supernatants obtained from stimulated sensitised lymphocytes. The following have been identified with some precision by their ability to modify the *in vitro* activity or behaviour of susceptible indicator cells: Migration Inhibitory Factor (MIF) which inhibits the movement of normal unsensitised macrophages (David et al., 1964; Bennett & Bloom, 1966), Chemotactic Factor which induces the directional movement of normal unsensitised macrophages along a concentration gradient of the material (Ward et al., 1969), Lymphotoxin (LT) which damages and kills susceptible target cells in a non-specific manner (Granger et al., 1969). The presence of such substances in active supernatants has been considered

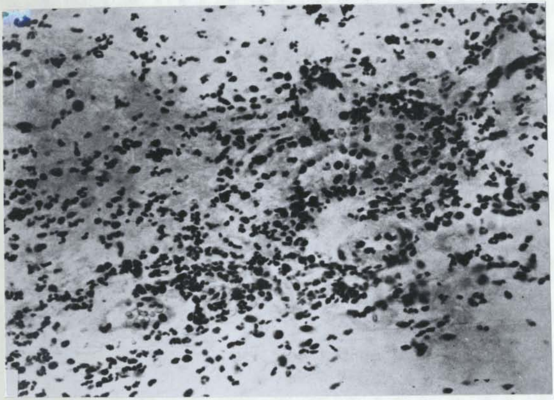


FIGURE 8. The skin of a normal guinea-pig 6 hours after the intradermal injection of 0.1 ml of supernatants of sensitised peritoneal exudate lymphocytes cultures with PPD, 10 ug/10^7 cells. Section shows the area between the dermis and panniculus carnosus. H & E stain, x 200. (Pick et al., 1969).

an *in vitro* correlate of the delayed hypersensitivity reaction (Lawrence, 1968).

Other factors have been described whose separate characteristics have been less well worked out, e.g., blastogenic factors that stimulate normal lymphocytes to transform and divide (Kasakura & Lowenstein, 1965; Bennett & Bloom, 1968; Dumonde et al., 1969; Valentine & Lawrence, 1969), or a factor that may inhibit the movement of normal peripheral blood polymorphonuclear leukocytes (Søborg, 1967, 1969).

There is thus a strong possibility that the skin reactive or inflammatory factor is simply a mixture of these factors. A complete answer to this must necessarily await the acquisition of more information regarding the physicochemical characteristics of SRF, as compared to MIF, chemotactic factor or LT. But it is most likely that these other factors are important in the development of the tuberculin reaction, since their *in vitro* presence appears to correlate so well with the ability to develop a delayed hypersensitivity skin reaction.

Thus Dumonde et al. (1969) were able to show that, in their experiments, the supernatants from stimulated lymphocyte cultures, as well as producing an inflammatory response upon injection *in vivo*, also had the ability to stimulate normal lymphocytes to synthesize DNA, i.e., had mitogenic activity, were cytotoxic to mouse fibroblasts and inhibited the movement of normal peritoneal exudate macrophages.

Bennett and Bloom (1968) fractionated their lymphocyte culture supernatants, after concentration by gel filtration, on Sephadex G-100 columns. They found a peak corresponding to an average molecular weight of approximately 67,000. Marked inhibition of macrophage migration was found in this fraction derived from stimulated lymphocyte cultures. It was this material that produced inflammatory reactions upon intradermal injection. These results would be against the SRF being an antibody or antigen/antibody complex.

Heise and Weiser (1969) similarly fractionated their culture supernatants, using Sephadex G-200 and found that a fraction, corresponding to the elution peak of bovine γ-globulin and part of a broad peak retarded by the column, contained most of the cytotoxic activity, as indicated by the inhibition of growth of mouse L cells and also inhibited macrophage migration and produced skin reactions upon intradermal injection.

It is therefore pertinent to examine the active supernatants injected to test for SRF, for the presence of MIF or other of the possible in vitro correlates of delayed hypersensitivity.

A further consideration is whether the cells actually producing the mediators are those lymphocytes that transform to blasts and subsequently divide. Indeed, it may be that the process of transformation is not inseparably linked to the production of mediators.

Bennett and Bloom (1967) and Krejci (1969) were able to show that MIF and SRF are detectable within 6 hr of incubation of sensitive lymphocytes with antigen, but this does not argue against the cells producing the mediators going on to full blast transformation. Indeed, it is unfortunate, considering the large cell and antigen concentrations used in the production of SRF that in none of the experiments so far considered was lymphocyte transformation actually assessed. This could be considered particularly relevant in the light of the numerous reports apparently demonstrating specific sensitised cell damage by tuberculin (vide supra). Especially may this be important where cultures are carried out under serum-free conditions. Heise and Weiser (1969) did demonstrate only a minor fall in cell viability as assessed by trypan blue exclusion, in serum-free cultures with PPD after 24 hr. However, those authors used much lower cell concentrations than did Pick et al. (1969), or Krejci et al. (1969). Indeed, Svejcar et al. (1968) commented, using the same cell culture system as Krejci et al. (1969) that most of the cells were non-viable by 18 hr of culture. Thus one might consider the production of SRF as a result of specific cell damage, which may be reversible, depending upon the culture conditions. Pick et al. (1969) used puromycin and actinomycin to block the release of SRF which suggests that active protein or RNA synthesis is required for the production of that material. However, it cannot be ascertained as yet whether the production of SRF can be linked to the proportion of cells that are undergoing transformation.

There is certainly some recent evidence suggesting that the production of MIF and the process of lymphocyte transformation as assessed by DNA synthesis may be dissociated. Thus, in some patients with chronic mucocutaneous candidiasis, their cells may respond in vitro to Candida antigens by normal thymidine incorporation into DNA, and yet be unable to make MIF (Rocklin et al., 1970; Valdimanson et al., 1970).

It has also been demonstrated that guinea-pigs sensitised to carbohydrate antigens of mycobacteria (Chaparas et al., 1970) or peptides of tobacco mosaic virus (Spitler et al., 1970) show positive delayed skin reactivity to these antigens, produced MIF or demonstrated inhibition of macrophage migration in vitro, and yet were not stimulated in either case to transform or incorporate thymidine.

Although these results may be interpreted as indicating a complete dissociation between the process of transformation and the production of soluble factors, it is probably safer at present to suggest that cells that begin mediator production are not irrevocably committed to a metabolic process resulting in eventual DNA synthesis. Thus, in disease states, the nature of the antigenic stimulus or even specific culture conditions might halt the process at any stage up to and including actual cell division, and of course, DNA synthesis is the parameter usually assessed when measuring transformation. A further possibility to be borne in mind is that the two processes might involve two different populations of cells.

However, these considerations need not detract from the value of assessment of in vitro transformation during the production of mediators. Indeed, these measurements might give valuable information regarding the possibility of a dissociation of these processes in the response of cells to tuberculin.

To turn now to the histological features of the inflammatory response induced by the injection of supernatants rich in SRF, it is to be noted that, in all descriptions so far, polymorphonuclear leukocytes constitute an important part of the inflammatory exudate, in addition to the mononuclear cells present (Bennett & Bloom, 1968; Dumonde et al., 1969; Krejci et al., 1969; Pick et al., 1969).

If the features of the SRF-induced reactions are compared to the histological descriptions of the tuberculin reaction itself, it can be seen that some polymorphonuclear leukocyte infiltration has been commented upon in the latter, although the degree of this contribution by granulocytes differs between authors (Dienes & Mallory, 1932, 1936; Laporte, 1934; Gell & Hinde, 1951, 1958; Wesslen, 1952; Boughton &

Spector, 1963). However, most investigators consider that mononuclear cells are the prime instigators of the tuberculin reaction and for this reason considerable attention has been paid to MIF as an in vitro correlate of the delayed hypersensitivity reaction. This is not unreasonable since the production of MIF in vivo at the site of the reaction would be considered likely to immobilize macrophages that had been induced to infiltrate the area. The importance of immune macrophages in actual resistance to facultative intracellular parasites such as mycobacteria has been commented upon frequently (see Mackaness, 1967). The initial movement of mononuclear cells into the region might be induced by the production of specific mediators released by sensitised lymphocytes such as Chemotactic Factor or by monocytes or macrophages themselves interacting with the antigen. Even so, the infiltration of polymorphs in the tuberculin reaction requires some explanation and this is usually suggested as being due to a non-specific response to tissue damage at the site of antigen administration (Boughton & Spector, 1963). Another possibility to be considered in the light of recent demonstrations of an effect of antigen-stimulated sensitive lymphocytes upon the movement of polymorphs in the Leukocyte Migration Test (Søborg & Bendixen, 1968; Søborg, 1968; Søborg, 1969; Clausen, 1970) is that the polymorphs are present because of the production of soluble factors by sensitised lymphocytes, in the same way that MIF and Chemotactic Factor appear to act upon monocytes and macrophages.

In addition, the presence of a significant proportion of polymorphs in the skin reactions induced by SRF, although this could be due to tissue damage, could also be the result of lymphocyte-produced soluble factors. Certainly the presence of substantial proportions of polymorphs in the reaction could contribute to the inflammatory response itself, especially in view of the demonstration and isolation of permeability factors in neutrophils (Ranadive & Cochrane, 1968). Thus, it would seem logical to examine the effects of SRF or the inflammatory supernatants upon polymorphs in the same way that their effect upon macrophages has been examined in previous work (Bennett & Bloom, 1968; Dumonde et al., 1969; Heise & Weiser, 1969).

If the tuberculin reaction would thus appear to be the result of an interaction of cells of the immune system and of the products of their metabolism, acting upon the small vessels and tissues of the skin, there remains a possible role for other inflammatory factors. Thus, although physicochemical evidence would suggest that the skin reactive factor produced by sensitised lymphocytes is different from the recognised pharmacological mediators of inflammation such as histamine, serotonin, kallikrein, or globulin permeability factor, it is still possible that it may act via one or more of these systems.

Finally, it should be stressed that all the work so far undertaken upon SRF has been in a guinea-pig or rabbit system and it would, therefore, be logical to attempt to demonstrate its production in man. A further consideration concerns whether or not it is possible to demonstrate a correlation between the degree of sensitivity of the donor, as assessed by skin reactivity, the response of the cells _in vitro_ as measured by parameters of transformation, and the production of SRF and other proposed mediators of the delayed hypersensitivity reaction.

SCOPE OF THE THESIS

The primary aim of this thesis was to demonstrate in man the production of skin-reactive (or inflammatory) factors (SRF) by sensitised lymphocytes stimulated by the antigen, tuberculin PPD. For practical reasons, this necessitated the development of a culture technique using peripheral blood lymphocytes as a putative source of the SRF.

Although experiments were begun using both guinea-pig and human donors as a cell source, there were early difficulties in demonstrating the presence of significant specific inflammatory activity in the supernatants of cultures of sensitive human peripheral blood lymphocytes stimulated with PPD. This led to a concentration of effort upon the development of a guinea-pig lymphocyte culture system that could later be applied to experiments in man. This seemed reasonable since the production of SRF by guinea-pig lymphocytes, albeit lymph node lymphocytes, was already fairly well established. In addition, since both human and guinea-pig supernatants were assayed by intradermal injection into normal guinea-pigs, there would not be the complication of possible species differences which could have accounted for the difficulties with the human system. However, considerable difficulties were experienced in the culture of the guinea-pig lymphocytes and it was some time before a system applicable for use in man was finally developed. Subsequently, attention was redirected back to the search for evidence for SRF production by human lymphocytes, using essentially the technique developed in the guinea-pig, with some modifications.

In addition, certain other parameters of the immune response were measured. *In vitro* assays of other potential mediators were carried out, the effects of inflammatory supernatants upon the normal migration of guinea-pig peritoneal exudate cells and human peripheral blood polymorphonuclear leukocytes being examined.

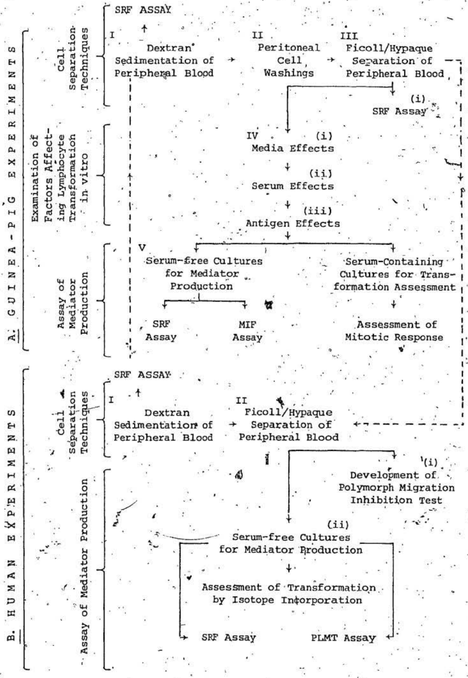
Lymphocyte transformation was assessed morphologically, by cell division or by the incorporation of radio-actively labelled precursor molecules such as leucine, uridine or thymidine into stimulated cells. This assessment seemed of some importance in the determination of the significance of transformation to the production of these soluble factors, the proposed mediators of the delayed hypersensitivity reaction.

Both the in vitro assay of mediators and the assessment of transformation acted in addition as an indicator of the functional activity of the cells in culture.

Finally, it was hoped that, at the end, some correlation might be found between the cutaneous reactivity of the donor to PPD and the in vitro response to antigen of the cells from that donor, as assessed by the methods discussed above.

In this thesis, the experiments with guinea-pigs will be discussed first, followed by the experiments using human cells. The stages in the development of the experiments are indicated diagrammatically in Figure 9. Following each section, there will be a short discussion and a general examination of the conclusions of the experiments will be presented at the end of the thesis.

FIGURE 9.


A. GUINEA - PIG EXPERIMENTS
Cell Separation Techniques
Examination of Factors Affecting Lymphocyte Transformation in vitro
Assay of Mediator Production
SRF ASSAY
I
Dextran Sedimentation of Peripheral Blood
II
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III
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(i)
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IV (i)
Media Effects
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V
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B. HUMAN EXPERIMENTS
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A. EXPERIMENTS USING GUINEA-PIGS

On the basis of the investigations of other workers in this field (Bennett & Bloom, 1968; Dumonde et al., 1969; Heise & Weiser, 1969; Pick et al., 1969) and the proposed aims of these experiments, discussed above, it was decided that certain requirements existed for any guinea-pig culture system that was developed. These were that when using such a system, one could (a) reproducibly transform sensitised lymphocytes with specific antigen, (b) obtain substantial numbers of lymphocytes without excessive contamination by other cell types, and (c) apply it for use in man.

Over the series of experiments, lymphocytes were obtained from guinea-pigs, using three different methods. These were:

I. Dextran sedimentation of peripheral blood

II. Peritoneal cell washings

III. Centrifugation of peripheral blood on a ficoll-sodium diatrizoate mixture, modified from the method of Böyum, 1967.

I. Experiments using guinea-pig lymphocytes obtained by Dextran sedimentation of peripheral blood.

These experiments investigated:

(a) the cell types found after Dextran sedimentation of guinea-pig peripheral blood

(b) the mean total number of peripheral blood lymphocytes obtainable by this method

(c) the ability of those lymphocytes to undergo transformation. Phytohaemagglutinin (PHA) was used initially in preference to PPD because of the substantial number of small lymphocytes that it stimulates to divide (Wilson & Thomson, 1968).

(d) the inflammatory activities of supernatants from cultures of sensitised lymphocytes stimulated with PPD as compared to those from control cultures, by intradermal injection into normal tuberculin-negative guinea-pigs.

MATERIALS AND METHODS

Animals: Male Hartley strain guinea-pigs weighing from 500-1000 g were used in these experiments.

Immunization: Guinea-pigs weighing from 500-600 g were immunized once with either complete Freund's adjuvant (CFA, Mycobacterium butyricum, 0.5 mg/ml, BBL; Becton, Dickenson & Co. Canada) 0.1 ml into each footpad, or with a suspension of 40 mg of freeze-dried BCG vaccine (Connaught Laboratories, Toronto, Canada) in 1 ml of saline and 0.5 ml CFA, 0.05 ml into each footpad. Control normal guinea-pigs received no immunization.

Skin Tests: Skin reactivity was assessed, approximately 4 weeks after immunization, by intracutaneous injection of tuberculin purified protein derivative (PPD; 1 tuberculin unit (TU), 5 TU or 250 TU in 0.1 ml; Connaught Laboratories) into the shaved abdominal skin. The reaction, which was maximal at 24 hr, was assessed by measuring the diameter of erythema and induration. Animals not reacting to the lowest dose of PPD were tested a few days later with a higher dose.

Preparation of Lymphocytes: Peripheral blood was obtained under sterile conditions by intracardiac puncture using an 18 G needle and a 30 ml syringe. Animals were anaesthetised with diethyl ether U.S.P.

The blood was anticoagulated with sodium heparin U.S.P. (Connaught Laboratories) at a concentration of 10 units per ml of blood. An equal volume of a 6% solution of Dextran T 110 (Pharmacia, Uppsala, Sweden) in saline was then added to the blood and the two were mixed thoroughly in the syringe. The dextran/blood mixture was then transferred to sterile glass culture tubes and was allowed to sediment at a 45° angle at 37° C for periods of up to 3 hr until a plasma layer, relatively clear of erythrocytes, was visible. The conditions for red cell sedimentation were arrived at in preliminary experiments using various proportions of dextran to whole blood, sedimented upright or at a 45° angle, at 4° C, room temperature or 37° C. Even so, red cell sedimentation was rarely complete at the time that the leukocyte-rich plasma layer was pipetted off. However, longer periods of sedimentation reduced the yield of lymphocytes.

The cells were then washed twice in cold Hanks' balanced salt solution (HBSS, BBL; Becton, Dickenson & Company, Canada, Ltd.) containing 10 units of sodium heparin/ml. The cells were spun down at 400 g for 5 min at 4° C. Finally, the cells were resuspended in a known volume of HBSS and a cell count by haemocytometer and cell viability by trypan blue exclusion was carried out (Holmberg, 1961; Black & Berenbaum, 1964; Ling, 1968). Viability was greater than 90%. Also at this time a differential count was made, either by smears of the cells on serum-coated slides stained with Giemsa (Fisher Scientific Co., Ltd., Montreal, Canada) after fixation with methanol, or by phase contrast microscopy using a Wild Inverted M40 microscope.

<u>Culture Conditions</u>: (Modified from Zweiman, 1967)

PHA stimulation: Cells were cultured at a concentration of

$0.5 - 1.0 \times 10^6$ leukocytes/ml in 5 ml of either Eagle's Minimal Essential Medium (MEM, GIBCO, Grand Island Biological Co., New York) or RPMI 1640 (GIBCO) containing 20% heat inactivated foetal calf serum (BBL) with penicillin (100 units/ml) and streptomycin (100 ug/ml) (GIBCO). In some experiments, other culture media such as Medium 199 or L-15 (GIBCO) were tried. Either no stimulant or PHA, 0.1 ml stock solution (Phytohaemagglutinin reagent grade, Wellcome Reagents Ltd., Beckenham, England), was added to the cultures. The cultures were incubated in sterile glass tubes, 100 x 13 mm (Corning Pyrex brand, Fisher Scientific Co., Ltd.) with loose-fitting caps at 37-37.5° C in a humidified atmosphere of 5% CO_2 in air. Triplicate cultures were incubated either for 48 hr or, more usually, for 72 hr.

PPD stimulation: Cells were cultured at a concentration of from $1 - 10 \times 10^6$ leukocytes/ml in 2 ml of RPMI 1640 containing 20% heat inactivated foetal calf serum with penicillin and streptomycin. Stimulated cultures had PPD (Connaught Laboratories) 1 ug or 10 ug/ml of medium added to them. Control cultures either received an equal volume of diluent, or the cells were killed by heating at 60° C for 30 min (viability was assessed by trypan blue exclusion) and the appropriate amount of antigen was added. A further control consisted of culture medium alone containing antigen. Cultures in glass culture tubes were incubated for periods of from 24-96 hr at 37° C in a humidified atmosphere of 5% CO_2 and air.

Assessment of Stimulation: At the end of the incubation period, PHA-stimulated cultures were harvested. Colchicine (Calbiochem) 0.25 ml/5 ml culture was added, giving a final concentration of 5×10^{-7} M. Five hours later, the cultures were centrifuged at 400 g for 5 min at room temperature. The supernatant fluid was removed and the cells were resuspended by gentle tapping in 4-6 ml of warm 1.0% sodium citrate and then incubated at 37° C for 20 min. The cells were centrifuged once more and the supernatant fluid was removed. The cell deposit was then resuspended by tapping and 4-6 ml of freshly prepared cold acetic alcohol (1 part glacial acetic acid, 3 parts absolute methanol) was added drop by drop. After centrifuging, the supernatant fluid was removed and a further 5 ml of acetic alcohol was added as before. After leaving a few minutes, the cells were centrifuged once more, most of the supernatant removed and the cells were resuspended in the drop or two of acetic alcohol that remained. A drop of this cell suspension was then placed upon a clean glass slide and the fixative was allowed to evaporate. The slides, when dry, were placed in buffered water (pH 6.8) for 20 min and then stained with Giemsa and subsequently mounted. The number of cells entering metaphase in the last 5 hours of culture (since colchicine was added) was assessed by counting the number of mitoses seen per 1000 mononuclear cells. Slides were scanned from end to end. Only well-spread metaphase plates were counted, where there could be no doubt about the presence of chromosomes. Either one or two slides were prepared from each culture.

<u>Preparation and Assay of SRF</u>: PPD-stimulated cultures and control cultures were centrifuged at 1000 x g for 10 min and the supernatants were removed. Smears of the deposited cells were stained with Giemsa after fixing and were mounted. Aliquots of the supernatants, 0.1 ml, were injected intradermally into the shaved abdominal skin of tuberculin-negative guinea-pigs. The inflammatory activity of the supernatants was assessed by measurements of the skin reactions produced over the

TABLE 1

Mitotic Response of Guinea-pig Lymphocytes to PHA

Total No. of Expts.	% Mean Differential Cell Count ± S.D.	Mean Total No. of Leukocytes	No. of Expts. with Mitotic Response	% Mean Mitotic Response	% Max. Mitotic Response	Range of Mitotic Response
	Polymorphs 31.5 ± 23.5					
10	Lymphocytes 70 ± 21.2	$53 \pm 36 \times 10^6$	2	1.6 ± 1.5	4.2	0.8 - 4.2
	Monocytes 5 ± 3					

Mitotic Response, as number of Mitoses/1000 mononuclear cells, expressed as %

next 24-48 hr. Reactions were assessed in terms of the diameter of the erythema or pallor produced, the approximate degree of erythema and the degree of induration.

RESULTS

Stimulation of lymphocytes from dextran-sedimented guinea-pig blood.

In only 20% of the experiments in this series was it possible to find evidence of stimulation of the lymphocytes in culture, to the point of actually producing cell division (Table 1). This variability from experiment to experiment was not related to such factors as foetal calf serum or culture medium, since the same batches of these were used throughout. It was also unlikely to be related to polymorphonuclear leukocyte contamination, since some experiments had less than 10% granulocytes in the final cell suspension and still cells were not found to divide. Experiments set up to test the effect of various doses of PHA from 1 ul-20 ul/ml of culture fluid were among those experiments that were unsuccessful.

Morphologically, some cultures consisted only of degenerating polymorphonuclear leukocytes and small lymphocytes, whilst others, not showing dividing cells, still had some indications of possible lymphocyte stimulation in the presence of large, palely staining pink nuclei, generally round or slightly indented (Figure 10). Cytoplasm was usually not visible in these slides, because of the technique used for preparation of the chromosome spreads.

Where stimulation had occurred, the mitotic response was generally low, the mean being only 0.3% per hr and the maximum being 0.8% per hr.

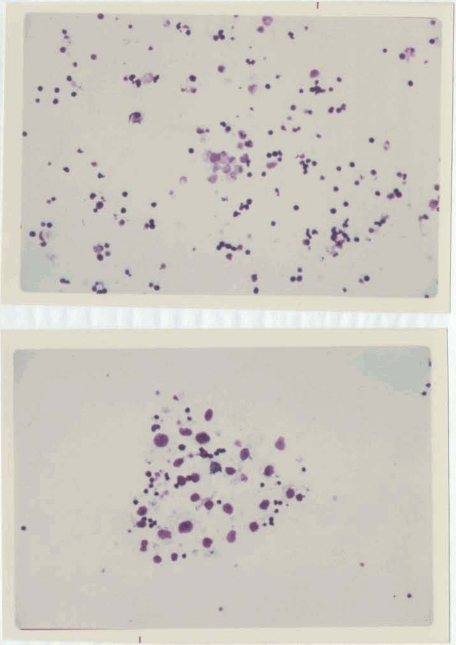


FIGURE 10. Upper: Small lymphocytes and larger pinkly stained nuclei in smears of cells from cultures of guinea-pig peripheral blood lymphocytes stimulated with PPD 1 ug/ml. Giemsa. x 160.

Lower: Smear of a cell culture in which blast transformation and cell division was occurring. Giemsa. x 160.

TABLE 2

Inflammatory Activity in Supernatants from Guinea-pig Lymphocytes Incubated for 48 hr with Antigen

Cell Concentration 10^6 cells/ml	No. of Expts.	Antigen Concentration ug PPD/ml	SRF Assay: Mean Diameter of Skin Reaction in mm ± S.D. (Range of Skin Reactions)			
			Stimulated	Unstimulated	Killed Cells	Medium
1×10^6	1	1	3	-	-	2
		10	2	-	-	2
2×10^6	2	1	4 (3-5)	5 (3-7)	1 (0-2)	-
	1	10	5	6	3	-
10×10^6	6	1	4.7 ± 2.7 (3-10)	2.0 ± 1.3 (0-3)	1.1 ±1.2 (0-2.5)	0.5 ± 0.8 (0-2)
	5	10	6.6 ± 5.1 (3-15)	3.2 ± 1.5 (1-5)	2.1 ± 1.7 (0-4.5)	0.8 ± 1.3 (0-3)
Students'		1 p value		<0.1	<0.02	<0.01
t. test		10 p value		<0.2	<0.1	<0.05

Statistics carried out on experiments at cell concentration of 10×10^6 cells/ml, comparing results from stimulated cultures with those from controls.

With regard to other parameters measured in these experiments, there was a considerable contamination of the lymphocytes with polymorphonuclear leukocytes at times (31.5 ± 23.5%). The total numbers of cells ($53 \pm 36 \times 10^6$) available by this method would probably ensure the use of cell concentrations approximately the same as that used by Heise and Weiser (1969), Bennett and Bloom (1969) and Pick et al. (1969), at least in some instances.

Assay of inflammatory activity in supernatants from cultured lymphocytes.

The diameters of the skin reactions indicated in Table 2 are of the maximum size attained during the period of observation. In general, reactions reached a peak at from 6-12 hr and faded subsequently. Induration and erythema, in these relatively small responses, were approximately equal in area. In some 25% of cases, pallor rather than erythema was seen, but these reactions were always only 3 mm in diameter or less and were found in all groups.

The inflammatory properties of these supernatants were thus in most instances only slight, but in the final group of experiments, using 10×10^6 cells/ml of culture, there was some difference between supernatants from purportedly stimulated lymphocyte cultures and those from controls, where transformation or activation of the lymphocytes should not have occurred. These differences were most marked when the controls consisted of culture medium or killed cells with antigen. The differences were also more significant when 1 ug PPD/ml was used to stimulate rather than 10 ug PPD/ml. These were supernatants from cultures incubated for 48 hr but longer periods of incubation, up to 96 hr, did not increase the size of the inflammatory responses produced by injection of supernatant, nor did it increase the differences between stimulated and control supernatants.

Stained smears from 48 hr cultures rarely showed evidence for blast transformation (Figure 10) and there appeared to be a considerable amount of cell death, as evidenced by cells with irregular small pyknotic nuclei with poorly stained cytoplasm or even gross karyolysis. Cultures at 96 hr appeared to contain mostly dead cells.

DISCUSSION

These preliminary experiments into guinea-pig SRF were initially disappointing from several aspects. Thus the inflammatory activity of the supernatants produced here by guinea-pig lymphocyte culture was in most instances only minimal and it had been difficult to demonstrate unequivocably a specific release of the inflammatory activity in response to antigen. However, it had been shown that the inflammation was not produced simply by materials released after acute cell death. If one looks at results of previous workers using unconcentrated supernatants (Dumonde et al., 1969; Pick et al., 1969; Krejci et al., 1969) then some assessment of the present results may be made. The size of the reactions and the significance of the difference between inflammatory activity in stimulated and unstimulated control supernatants cannot be ascertained from the experiments of Dumonde et al. (1969). But in the results of Pick et al. (1969) the size of reactions produced by supernatants from stimulated cultures were about twice those from unstimulated control supernatants and, overall, the reactions were about twice the size of the reactions produced in the present experiments. However, there was, in Pick's experiments, also a considerable overlap between the size of the inflammatory reactions in the stimulated and control groups and the significance of the difference was not commented upon. In the experiments of Krejci et al. (1969), the reactions caused by stimulated culture supernatants were, at their peak at 24 hr, about 8 mm in diameter (mean area for the experiments was 68.2 sq mm), whereas the reaction to unstimulated culture supernatants had faded by then.

The peak of the inflammatory reactions produced in the present experiments occurred in the same period of time as that described by Bennett and Bloom (1969), Dumonde et al. (1969) and Heise and Weiser (1969), i.e., from 6-12 hr, but was later than the peak for Pick et al. (1969) and earlier than that for Krejci et al. (1969) — see Table A.

The small size of the reactions produced by the supernatants in this series of experiments could be due to too few cells being used in the initial cell inoculum, or by a failure of transformation of the stimulated lymphocytes, if transformation is necessary for the production of SRF. Certainly the concentration of lymphocytes used in these experiments was lower than that used by Dumonde et al. (1969), Pick et al. (1969) and by Krejci et al. (1969). But against this, one has to balance the effect of high cell concentration upon the lymphocyte response to antigen, for although cell death is not a likely cause for the production of inflammatory factors, it would prevent active synthesis of inflammatory factors, obscuring the specificity of SRF release. In addition, the presence of large numbers of polymorphonuclear leukocytes could also obscure antigen specific release of inflammatory factors by non-specific release of materials inciting an inflammatory response (Ranadive & Cochrane, 1968).

If non-specific inflammatory activity can be reduced by using lymphocyte populations with less contamination from other cell types, and specific release of SRF can be increased by the use of culture conditions optimal for cell stimulation and survival, then the problems of low total amounts of the inflammatory factors could be overcome by the use of physicochemical techniques of concentration. However, this leaves unanswered the difficulties that were encountered in inducing transformation with PHA in this series of experiments, using cell concentrations much lower than those used for the production of SRF. Indeed, it is probable that failure of transformation of the sensitised lymphocytes by antigen was itself responsible for the somewhat equivocal results obtained in the SRF experiments.

The difficulties inherent in the guinea-pig culture system have been commented on before (Marshall & Roberts, 1963; Knight et al., 1965; Phillips & Zweiman, 1970). However, it was decided at this time to examine another source for guinea-pig lymphocytes, i.e., from the peritoneal cavity, and to investigate the problems of inducing transformation by PHA and PPD using these cells.

II. Experiments using guinea-pig lymphocytes obtained by washing cells from the peritoneal cavity.

These experiments investigated:

(a) the cell types found in peritoneal cavity cell washings (PCWs) from normal healthy guinea-pigs

(b) the mean total number of such cells obtainable by this method

(c) the ability of lymphocytes obtained by this means to undergo transformation. PHA was used to investigate this response in the majority of experiments.

(d) the effects of some variations in culture conditions, e.g., serum effects, antibiotic effects, were also investigated.

MATERIALS AND METHODS

Animals: Male Hartley-strain guinea-pigs weighing from 500-700 g were used in these experiments.

Immunization: Most of the animals used had received no immunizations. Those used in experiments with PPD were immunized with a BCG/FCA mixture as in Section I.

Skin Tests: Skin reactivity was assessed as described in Section I.

Preparation of Lymphocytes: The animal was exsanguinated by intracardiac puncture under ether anaesthesia and the blood obtained was allowed to clot in a sterile glass 15 x 125 mm culture tube (Corning). The heat-inactivated serum from this was used later in the lymphocyte cultures. Immediately after exsanguination, Hanks solution (HBSS), 50 ml at 4° C, was injected intraperitoneally under sterile conditions, and the abdomen of the animal was massaged for 10 minutes. The cell suspension was then drained off through a sterile 14 G plastic catheter (C.R. Bard, Inc., Murray Hill, N.J.) into a sterile glass bottle kept cold in ice. The cells were washed twice in cold HBSS with 20% foetal calf serum (FCS) at 400 g for 5 min and were finally resuspended in a known volume of HBSS. A cell count was performed using a haemocytometer and cell viability was measured by trypan blue exclusion. The cell differential count was assessed by a smear of the cells on a serum-coated slide, later to be fixed in methanol and stained with Giemsa, and by phase-contrast microscopy, after incubation of the cells for 30 min with a mixture of FCS and latex particles in saline (Bacto-latex 0.81, 1:25 dilution of the stock suspension, Difco Laboratories, Detroit, U.S.A.).

Culture Conditions: Cells were cultured at a concentration of 0.5 - 1.0 x 10^6 leukocytes/ml in 1.6 ml of RPMI 1640 (GIBCO) and 0.4 ml of heat-inactivated autologous serum (incubated for 30 min at 56° C). In one experiment, homologous and heterologous (human cord) sera were also used. In early experiments, the culture medium contained penicillin 100 u/ml and streptomycin 100 ug/ml but later these were supplemented with kanamycin 100 ug/ml (GIBCO) after experiments investigating the influence of this antibiotic on the culture conditions. PHA (Wellcome) at a concentration of 2.5 ul stock solution per ml of culture in 0.1 ml was used in most experiments, after dose/response experiments indicated this to be optimal for stimulation of the lymphocytes. Control cultures received instead 0.1 ml saline per culture. In one experiment, PPD (Connaught) at a concentration of 2.5 ug/ml culture was also used to stimulate cell division. Cultures were incubated in sterile glass culture tubes, 100 x 13 mm with loose-fitting caps at 37-37.5° C in a humidified

TABLE 3

Transformation of Guinea-pig Lymphocytes from Peritoneal Cell Washings Stimulated with PHA

Total No. of Expts.	% Mean Differential Cell Count ± S.D.		Mean Total No. of Leukocytes	No. of Expts. with a Mitotic Response	% Mean Mitotic Response	% Max. Mitotic Response	Range of Mitotic Response
	Polymorphs	9 ± 6.9					
21	Lymphocytes	74 ± 17.6	$24 \pm 29 \times 10^6$	9	1.3 ± 0.23	3.6	0.17 - 3.6
	Monocytes	18 ± 11.8					

atmosphere of 5% CO_2 in air. Cultures were incubated in triplicate for 72 hr after PHA stimulation and for 120 hr after PPD.

Assessment of Stimulation: This was carried out using 5 hr colchicine block (0.4 ug per culture) as described in Section I.

RESULTS

Stimulation of lymphocytes from peritoneal cell washings with PHA or PPD.

In this series of experiments, the majority of the cells obtained from peritoneal washes were mononuclear leukocytes; polymorphonuclear leukocytes made up only about 9% of the cells, most of these being eosinophils (see Table 3).

Overall, the technique resulted in a mean yield of cells per animal approximately half that obtainable by dextran-sedimentation of the peripheral blood.

Initial experiments (Figure 11) suggested that the optimal concentration of PHA for use to stimulate lymphocyte division in this system was 2.5 ul stock PHA/ml culture, which was approximately that indicated by the results of Phillips and Zwieman (1970) who also used guinea-pig lymphocytes, but obtained from peripheral blood. This concentration of PHA was therefore used in subsequent experiments.

In addition, it was demonstrated that transformation of sensitised guinea-pig lymphocytes could be induced by PPD at a concentration of 2.5 ug PPD/ml. The degree of transformation as assessed by the mitotic response was quite variable from culture to culture, and the mean response from triplicate cultures was only 0.9% after colchicine block for 5 hr.

The effect of kanamycin on transformation: Early in this series of

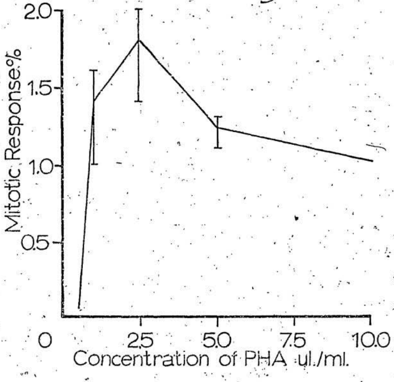
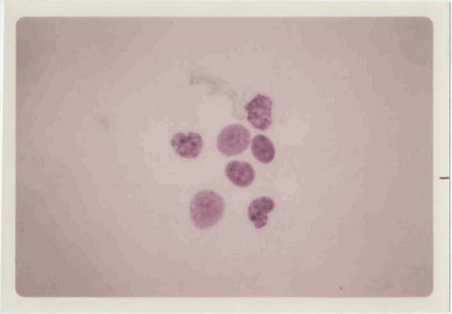


FIGURE 11. The effect of PHA concentration on the mitotic response of guinea-pig lymphocytes from peritoneal washings. The range of results in triplicate cultures is shown.



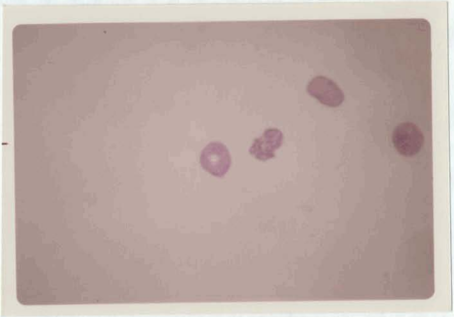


FIGURE 12. Cells from smears of cultures in which blast transformation did not occur, demonstrating chromatin clumping and nuclei with large or small holes. Giemsa. x 1000.

experiments, when slides were being examined for the presence of mitotic figures, it was noticed that some of the stained nuclei had large or small holes in them, whilst others exhibited masses of clumped chromatin (Figure 12). The majority of the nuclei, however, which were from cultures with a low or absent mitotic response, consisted of either large, pink, faintly stained nuclei (Figure 10) or small, darkly staining nuclei. Hayflick (1969) in a description of <u>Mycoplasma pulmonis</u> infection in tissue culture, had described the cytological changes that occur in the presence of this organism, namely "leopard" nuclei in which masses of clumped chromatin occur intranuclearly, and fragmented nuclei and nucleolar haloes. Because <u>Mycoplasma</u> has been found in guinea-pigs (Burdon-Williams, 1968) in the respiratory and intestinal tracts, the possibility that an infection with these organisms might be interfering with the process of transformation was considered. This was suggested, in part, by the morphological changes and also by the fact that cultures in this series and in Series I were seen where transformation appeared to have been halted before cell division occurred, and breakdown of the stimulated cells then followed. For these reasons, a trial of kanamycin 100 ug/ml of culture was undertaken.

When the Mitotic Response in cultures containing kanamycin was compared with that in cultures containing a combination of penicillin and streptomycin, it was seen that the response was approximately doubled (Table 4). This difference was highly significant. In an experiment where kanamycin alone was compared with a mixture of all three antibiotics, the mitotic responses were similar. As a result of these findings, kanamycin at a concentration of 100 ug/ml was used in

TABLE 4

Transformation after Stimulation with PHA 2.5 ul/ml in Cultures with Variations in Antibiotic and Serum Content

Serum Content	Mitotic Response % ± S.D. Kanamycin	Penicillin and Streptomycin	Kanamycin Penicillin and Streptomycin
Autologous	2.02 ± 0.33	1.03 ± 0.24	1.63*
	$p < 0.001$		
Homologous	1.16		
Heterologous	0.96		

* Mean of triplicate cultures

all subsequent experiments using guinea-pig lymphocytes.

Autologous serum was used in these experiments since it was hoped eventually to use the culture system in a series of experiments correlating an *in vivo* manifestation of immunity, i.e., skin reactivity to tuberculin with the *in vitro* production of inflammatory factors and serum might contain factors modifying the *in vivo* inflammatory response. But it is also interesting to note the lower mitotic response seen in cultures containing homologous or heterologous sera compared with that attained with autologous serum (Table 4).

The mean mitotic response obtained in the experiments using lymphocytes from peritoneal cell washings was 1.3% whilst the maximum mitotic response was 3.6%, as indicated in Table 3. Following the introduction of kanamycin, in a series of seven successful experiments, the mean mitotic response was 1.9%. However, after this the lymphocyte cultures persistently failed to transform under the influence of PHA and no definite cause could be found for this. Indeed, alterations made in culture conditions, materials or technique did not persistently improve the response. Harvested cultures after colchicine block contained degenerating small lymphocytes and there was little evidence of transformation.

DISCUSSION

This system, using guinea-pig lymphocytes obtained by washing the peritoneal cavity, was introduced because of the problems prevailing in the guinea-pig peripheral blood lymphocyte cultures. Initially it appeared that the serious drawbacks of this method, i.e., lack of application to man and the relatively low numbers of cells obtainable, would be outweighed by the reliability of the PHA-induced transformation and the low polymorphonuclear leukocyte contamination. However, with the subsequent persistent failure to find transformed and dividing cells in the lymphocyte cultures it became clear that this technique would not be satisfactory in its present form. It may be that the main disadvantage to this method of obtaining guinea-pig lymphocytes is the inherent risk of contamination by bacteria or Mycoplasma, although this was not apparent in numerous experiments that have since been conducted using guinea-pig peritoneal exudate cells. However, it would appear that contamination is the most likely explanation for the kanamycin effect seen above and possibly also for the persistent failure of cultures at the end of the experiments. Thus, even if the sterile technique used in the harvesting of the cells was not at fault, it is still possible that after exsanguination of the animal organisms might enter the peritoneal cavity from the gastro-intestinal tract. But experiments undertaken to demonstrate the occurrence of this phenomenon failed to do so, i.e., sterile (as assessed by agar plate or broth culture) peritoneal cell washings were obtained for up to 20 min following the death of the animal. Of course, one would not expect to demonstrate the presence of micro-organisms such as Mycoplasma by this technique. However, the use of fresh kanamycin in the cultures did not improve the outcome of the experiments in terms of the successful mitotic response of the lymphocytes to PHA.

So, after carrying out a series of twenty-one experiments, the final ten of which had ended in failure, it was decided that a return to peripheral blood as a source of lymphocytes would be made, using a technique for preparation of the cells different from that used in I.

III. Experiments using guinea-pig lymphocytes obtained by centrifugation of peripheral blood on a ficoll-Hypaque mixture.

The sedimentation rate of uniform spherical particles suspended in a liquid medium depends upon:

(a) particle size and density

(b) density and viscosity of the suspending medium.

The sedimentation of non-spherical particles, such as blood cells, is determined by these same factors and by the degree of deviation of the shape of the cells from the spherical. Red cells are small, but have a higher density than white cells (Reznikoff, 1923, Vallee et al., 1947) and may be aggregated by high polymer cellulose derivatives such as dextran (Skoog & Beck, 1956) or ficoll (Richter, 1963); erythrocytes thus sediment faster than leukocytes in the presence of such agents. The fact that the densities of some red cells show rather great deviations from the average value (Danon & Marikovsky, 1964) may explain the difficulties encountered using a simple dextran sedimentation method for guinea-pig peripheral blood in Section I.

Cell size and hence density depend upon the composition of the suspending medium: granulocytes are more sensitive to osmotic changes than are lymphocytes, in the sense that the density of granulocytes increases more than the density of lymphocytes in hyperosmotic surroundings (Böyum, 1968). Further, if the density of the suspending medium is close to the density of the cells, the sedimentation rates of the cells, relative to each other, are more influenced by their densities.

In 1968 Böyum introduced a technique for the isolation of mononuclear cells and granulocytes from human peripheral blood, based upon the principles discussed above. Diluted anticoagulated peripheral blood was floated upon a cushion of ficoll (a sucrose polymer) and Isopaque (an X-ray contrast medium, sodium diatrizoate) which was slightly hyperosmolar with respect to plasma (1.12 plasma equivalents). Red cell aggregation and increasing granulocyte density at the interface produced, on centrifugation at 400 g, the deposition of erythrocytes and granulocytes as a fraction in the ficoll-Isopaque and a layer of mononuclear cells at the interface. Subsequently, after removal of the mononuclear cells, granulocytes could be recovered by sedimentation of the erythrocyte fraction with dextran under the force of gravity alone.

This technique was modified in the experiments described in this section for use with guinea-pig peripheral blood. The following were investigated:

(a) the degree of separation of the cell types found in guinea-pig blood

(b) the total yield of mononuclear cells obtained by this method

(c) the ability of the lymphocytes obtained to divide on stimulation by PHA and PPD.

MATERIALS AND METHODS

Animals, Immunization and Skin Testing: These were as described in Sections I and II.

Preparation of Lymphocytes: The animal was exsanguinated by intracardiac puncture under ether anaesthesia using sterile technique. If autologous serum was required for the lymphocyte cultures, the blood was withdrawn into a 30 ml plastic disposable syringe (Jelco Laboratories, New Jersey, U.S.A.) and after exsanguination was complete, 8-10 ml of the blood was transferred to a sterile plain glass tube and left to clot. Immediately after this transfer, 2.7% EDTA (ethylene-diamine-tetra-acetic acid in distilled water, pH adjusted to 7.4 with NaOH) 1 ml per 20 ml of blood, was added to the syringe and mixed thoroughly with the blood. Serum was prepared by loosening the clot from the sides of the tube with a sterile glass rod, centrifuging at 2000 g for 20 min at room temperature and heat-inactivating at 56° C for 35 min in a water bath. If autologous serum was not required, 1.5 ml of the 2.7% EDTA was placed in the syringe before undertaking exsanguination, since 25-30 ml of blood was usually obtained from each animal. The anticoagulated blood was then transferred to a sterile 75 cm^2 plastic flask (Falcon Plastics; Becton, Dickenson & Co. Canada) and an equal volume of sterile isotonic saline was added to it.

The separation fluid used consisted of a mixture of 24 parts ficoll (Pharmacia; Uppsala, Sweden, 9% w/v in distilled water) and 10 parts sodium diatrizoate (Hypaque sodium; Winthrop Laboratories, Aurora, Canada; 50% w/v in distilled water, as obtained commercially). This mixture had a final density, prior to autoclaving, of 1.098 g/ml. The sterile separation fluid was added, at room temperature, in 10 ml aliquots to each of two sterile round bottom glass centrifuge tubes, each with a plastic screw cap. The diluted blood was layered carefully onto the ficoll/Hypaque cushion by running the blood gently down the side of the tube from a Pasteur pipette. The blood was not allowed to mix with the separation fluid. By the time that the diluted blood had been layered onto each of the ficoll/Hypaque cushions, aggregates of red cells were already sedimenting into the mixture. The tubes were then

FIGURE 13. The separation of guinea-pig peripheral blood using the ficoll-Hypaque technique of Böyum (1968). The bottom fraction contains erythrocytes and polymorphs. This is followed by the clear separating fluid and at the interface between this and the diluted plasma can be seen the mononuclear cell layer.

carefully balanced and centrifuged at room temperature (18-20° C) at 400 g at the interface, for 35-40 min. At the end of that time, erythrocytes were deposited as a compact red bottom fraction, whilst an upper fraction was visible as a whitish layer at the interface (Figure 13). The clear plasma/saline was pipetted off down to within a few mm of the upper fraction and was discarded. Next the fraction at the interface was removed with a Pasteur pipette, which usually necessitated taking off some of the ficoll/Hypaque mixture also. To remove completely this layer it was necessary to move the pipette over the whole cross area of the tube. This fraction was diluted with HBSS containing heparin sodium 10 units/ml to give a final volume of about 40 ml, i.e., diluted approximately 1 to 4. The cell suspension was then centrifuged at 500 g for 16 min at 4° C in a conical or round-bottom, sterile, plastic-capped centrifuge tube with a capacity of 50 ml. The supernatant was discarded at the end of this time and the cell button was resuspended using a Pasteur pipette in a known volume of HBSS. Often the cells were quite difficult to resuspend at this stage. A cell count was performed using a haemocytometer and viability was assessed by trypan blue exclusion (see above). A differential count was made using stained smears of the cell suspension and later by phase-contrast microscopy after incubation of some of the cell suspension with latex particles and heat-inactivated foetal calf serum.

Culture Conditions: In studies of transformation, mononuclear cells were cultured at a concentration of 0.4×10^6 cells/ml for PHA stimulation and at $1 - 10 \times 10^6$ cells/ml for PPD stimulation, in 2 ml of RPMI 1640 (GIBCO) containing 20% heat-inactivated guinea-pig serum, which was pooled serum in dose/response experiments and autologous serum otherwise. The culture medium itself contained penicillin 100 units/ml, streptomycin 100 ug/ml and kanamycin 100 ug/ml. Phytohaemagglutinin (Wellcome) was diluted in saline and was added in 0.1 ml aliquots to each culture giving concentrations of 0.05 - 100 ul stock PHA/ml of culture medium. PPD (Connaught) was added as 0.1 ml per culture to give concentrations of 0.25 - 25 ug/ml culture medium. Triplicate cultures were incubated in sterile glass culture tubes, 100 x 13 mm, with loose fitting caps in 5% CO_2 in air at 37-37.5° C in an humidified atmosphere for 72 hr for PHA stimulation and for 120 hr for PPD stimulation.

Assessment of Stimulation: The technique of a 5 hr block of cell division at metaphase using colchicine (0.4 ug per culture) and subsequent harvesting was described in Sections I and II.

RESULTS

Stimulation of lymphocytes from ficoll/Hypaque separated guinea-pig blood.

In this series of experiments, it can be seen from Table 5 that the cell population obtained consisted almost entirely of mononuclear cells, the majority being classified as small and medium-sized lymphocytes on the basis of their staining characteristics and their appearance on phase-contrast microscopy with a characteristic size and shape, cytoplasm free of vacuoles and small in amount, unable to phagocytose latex particles and with a typical form of movement.

Overall, in this small initial series, the technique resulted in a mean yield of cells per animal of 36 ± 35 x 10^6 cells, rather more than the yield from peritoneal cell washings (Table 3). The yield

TABLE 5

Transformation of Guinea-pig Lymphocytes from Peripheral Blood Stimulated with PPD 2.5 ug/ml

Total No. of Expts.	Mean % Differential Cell Count ± S.D.		Mean Total No. of Leukocytes	No. of Expts. with Mitotic Response	Mean Mitotic Response (%)	Max. Mitotic Response (%)	Range of Mitotic Response
	Polymorphs	0.2 ± 0.4					
7	Lymphocytes	84 ± 7	$36 \pm 35 \times 10^6$	5	2.8 ± 1.6	5.3	1.4 - 5.3
	Monocytes	16 ± 7					

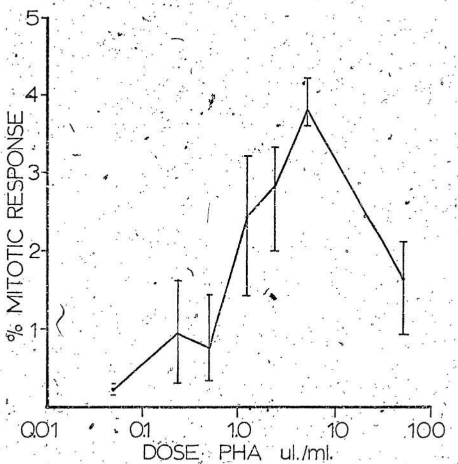


FIGURE 14. The effect of PHA concentration on the mitotic response of ficoll/Hypaque separated peripheral blood lymphocytes from guinea-pigs. The range of results in triplicate cultures is shown.

also compared well with the yield of mononuclear cells from dextran-sedimented blood (Table 1) which was around 40 x 10^6 cells, since there was only an insignificant contamination with polymorphonuclear leukocytes. In addition, the mononuclear layer on ficoll/Hypaque contained the majority of the platelets found in the blood sample. At times these platelets completely surrounded cells, predominantly monocytes, so they became enmeshed in a mass of platelets. The degree to which this occurred appeared to depend upon the ease with which the original sample of blood was obtained.

Initial experiments to find the optimal concentration of PHA for stimulation of these cells indicated a value of 5 ul stock PHA/ml of culture (Figure 14) which is close to that found in the experiments in Section II (Figure 11).

The dose of PPD found to be optimal for transformation was 2.5 ug PPD/ml (Figure 15) and this amount of antigen was used subsequently in the experiments. It was noted that the mitotic response with 25 ug PPD/ml was approximately the same as that obtained with 2.5 ug PPD/ml. However, as assessed by the staining characteristics of the smeared cells, there appeared to be more degenerating cells in the former (60%) than the latter (30%). This leads to difficulties in the interpretation of the mitotic response or rate since only nuclei with a healthy-stained appearance, i.e., a regular smooth outer membrane with evenly well stained nuclear contents, are included in the 1000 mononuclear cells counted. If responding cells tend to survive in cultures where some toxic process is occurring, e.g., in the presence of excess antigen containing preservative, this will artificially increase the % mitotic response in these cultures in comparison with cultures with a greater

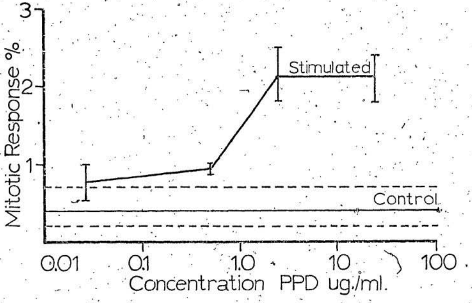


FIGURE 15. The effect of tuberculin concentration on the mitotic response of lymphocytes from ficoll/Hypaque separated peripheral blood from sensitised guinea-pigs. The range of results from triplicate cultures is shown.

non-responding cell survival.

Related to this, experiments using the optimal dose of PPD, 2.5 ug/ml of culture, with mononuclear cell concentrations of 1, 5 and 10×10^6 cells/ml, indicated that the lowest cell concentration appeared optimal with regard to both mitotic response and cell survival in culture as assessed by the staining characteristics of the cells.

In addition, it was found that cells transformed only in autologous or homologous sera and there were no mitoses seen in cultures containing 20% foetal calf or 20% human cord serum.

TABLE 6

Effect of Cell Concentration and Serum upon Transformation with PPD 2.5 ug/ml

	Mitotic Response % (% Degenerating Cells		
Cell Concentration /ml	Homologous Serum	Foetal Calf Serum	Human Cord Serum
1×10^6	2.1 % (30%)	0 (>90%)	0 (>90%)
5×10^6	0.47% (78%)		
10×10^6	0.7 % (74%)		

DISCUSSION

On the basis of the results obtained in these preliminary experiments with ficoll/Hypaque, it was decided to use this technique for the further investigation of SRF.

One minor problem that arose during the initial manipulations with ficoll/Hypaque was caused by the autoclaving of the separation fluid. With a freshly prepared solution, the mononuclear cell band did not cross the interface during centrifugation, but with autoclaved material the cell layer did enter the separation fluid although initially only partially. But within a single batch of ficoll/Hypaque, autoclaved and stored frozen, the final position of the band was closer to the red cell mass the longer the period of time that the separation fluid used had been stored. It was considered likely that autoclaving the ficoll modified the polymer in some way, possibly by hydrolysis, and this change appeared to increase with time. As the white cell layer approached closer to the red cells, it became increasingly difficult to remove all the band without producing significant red cell and polymorphonuclear leukocyte contamination. This problem was overcome by subsequently sterilising the ficoll/Hypaque by filtration as will be indicated in the following section.

For the moment, the significant proportion of monocytes in the cell preparations obtained using ficoll/Hypaque was not considered a serious disadvantage, even though the work of Heise and Weiser (1969) suggested that macrophages may produce non-specific inflammatory substances themselves. In part, this decision was related to recent demonstrations of the requirement of phagocytic cells (macrophages) for _in vitro_ transformation of sensitised lymphocytes by specific antigen (Oppenheim et al., 1968; Hersh & Harris, 1968; Seeger & Oppenheim, 1970).

The problem of the excessive numbers of platelets present in these preparations has already been commented upon. These particles at times completely encased monocytes in a platelet mass but the presence of these aggregations could not be directly linked to an inhibitory effect upon the process of transformation. Potentially more serious could be production of non-specific inflammatory factors from the breakdown of platelets.

A further difficulty unresolved and not related to the present technique of cell separation concerned the cell concentrations to be used in culture for the investigation of SRF. It appeared that actual lymphocyte transformation occurs in response to PPD more readily and with less cell death at low cell concentrations ($\approx 1 \times 10^6$/ml) whilst rather high concentrations of cells (1×10^7/ml or more) are required to produce mediators in sufficient concentrations for assay _in vivo_ using unmodified supernatants. Two possible answers to this problem may be examined. One involves the concentration of cell-free supernatants from stimulated cultures containing low numbers of cells ($1 - 2.5 \times 10^6$/ml). The simplest means by which this may be achieved is to culture the cells in serum-free medium and concentrate the supernatants by lyophilization after dialysis, as was carried out by Bennett and Bloom (1968) and Heise and Weiser (1969). This introduces immediately the question of the relationship of the immune response occurring _in vivo_, _in vitro_ in the presence of serum and _in vitro_ in serum-free medium. Are the responses of lymphocytes in each of these situations directly comparable? If one's sole interest is in the lymphocyte and its reaction to antigen, this problem may be only of minor consideration. But if it is desired, as here, to correlate an _in vitro_ event with _in vivo_ reaction, then it is of considerable importance, since other factors such as serum may modify the response of the lymphocyte to specific antigen. Of course, it is possible to culture the lymphocytes in autologous serum and subsequently separate active fractions from contaminating proteins by the use of techniques such as gel filtration (Dumonde et al., 1909) but it was hoped to avoid this at least initially. A further means by which cell-free supernatants might be obtained with a sufficiently high concentration of inflammatory factors, is to utilise a culture system similar to that introduced by Marbrook (1967). Here cells at a relatively high concentration in a small volume of culture fluid within a dialysis membrane are surrounded by a large reservoir of medium for the exchange of nutrient materials and the products of metabolism. Since MIF and SRF appear to be non-dialysable (Bennett & Bloom, 1968; Heise & Weiser, 1969; Pick et al., 1969) it was considered that this system might result in the production of relatively high concentrations of SRF within the confines of the dialysis membrane. It was resolved that an examination of the use of the Marbrook system for the production of SRF would be undertaken initially and the limited number of experiment where this was used are discussed in the section immediately following [III(i)].

III (i) Experiments on guinea-pig SRF using the Marbrook System.

MATERIALS AND METHODS

Animals, Immunization and Skin Testing: The animals used were male Hartley guinea-pigs that were immunized and subsequently skin tested as described in Sections I and II.

Preparation of Lymphocytes: The technique used was the same as that discussed in the previous section, except with regard to the preparation of the separation fluid. This consisted of a mixture of 24 parts ficoll (Pharmacia, 9% in distilled water) and 10 parts sodium diatrizoate (Hypaque sodium, Winthrop Laboratories, 37.5% w/v in distilled water). This mixture had a final density of 1.08 g/ml and was sterilized by filtration through a cellulose filter of pore size 0.22 u (Millipore Ltd., Montreal, Canada). The ease of filtration was considerably increased by the use of a glass fibre prefilter (Millipore Ltd.) superimposed directly on the bacterial filter in a single filter holder. Separation fluid was prepared in 300 ml lots and stored at -20° C in 10 ml aliquots for subsequent use. Prior to use it was important to warm the ficoll/Hypaque to room temperature and mix it thoroughly. Apart from this modification the preparation of lymphocytes proceeded along the lines described in Section III.

Culture Conditions: Cells for the assessment of transformation were cultured at a concentration of 1 x 10^6 mononuclear cells/ml in 2 ml RPMI 1640 with 20% heat-inactivated autologous guinea-pig serum. The culture medium contained penicillin 100 units/ml, streptomycin 100 ug/ml and kanamycin 100 ug/ml. PPD (Connaught) to give a concentration of 2.5 ug PPD/ml of culture was added as 0.1 ml per culture or 0.1 ml saline was added to controls. Sterile 13 x 100 mm culture tubes were used, being incubated in triplicate in 5% CO_2 and air at 37° C for 120 hr. For SRF production, the apparatus used is depicted in Figure 16.

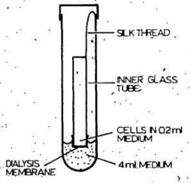


FIG. 16. Culture system used for SRF production.

It consisted of a sterile glass tube 75 mm long and 6 mm internal diameter, grooved at each end. At one end a dialysis membrane was stretched over and held in place by a nylon thread and a polythene cuff. This part of the apparatus was sterilised by autoclaving at 10 lb in^{-2} for 15 min. Such treatment did not modify the membrane such that it would allow the passage of chymotrypsinogen (M.W. 25,000) from inner to outer compartment over a period of 24 hr incubation at 37° C and thus it was unlikely that soluble mediators like MIF or SRF (M.W. >35,000) would be lost (Bennett & Bloom, 1968; Heise & Weiser, 1969; Pick et al., 1969).

Cells were placed within the inner tube to rest upon the membrane at concentrations equivalent to 7.5 - 25 x 10^6 cells/ml in a volume of 0.2 ml RPMI 1640 with 20% heat-inactivated autologous serum. The medium also contained the equivalent of 2.5 ug PPD/ml, whilst in the controls no PPD was added. After the addition of the cell suspension, the tube was introduced, by means of a piece of nylon thread tied around its upper end, into a sterile plastic 15 x 125 mm screw capped culture tube (Falcon Plastics) containing 4 ml of RPMI 1640. The tube was lowered until the dialysis membrane was beneath the surface of the surrounding medium and the levels inside and outside the tube were the same. The tube was fixed in that position by anchoring the nylon thread with adhesive tape, and the top was replaced on the culture tube. Cultures were incubated in 5% CO_2 and air in a humidified atmosphere at 37° C for 48 hr. At the end of the incubation period, the supernatants were withdrawn from the inner tube with a Pasteur pipette and were centrifuged at 400 g for 5 min in sterile 6 x 50 mm culture tubes (Kimax, Fisher Scientific Co. Ltd., Canada). Cell-free supernatants were then ready for injection for assay of SRF.

Assessment of Stimulation: This was carried out using 5 hr colchicine block (0.4 ug/culture) as previously described.

Assay of SRF: This was performed by injecting 0.05 ml of the cell-free supernatants of PPD-stimulated cultures and controls intradermally into the shaved abdomen of normal guinea-pigs. Reactions were assessed with regard to erythema (E) or induration (I) or pallor where that occurred.

RESULTS

SRF Assay using supernatants from dialysis tube cultures.

As indicated in Table 7, only in experiment 2 was there a signi-

TABLE 7

SRF Assay Using Supernatants from Dialysis Tube Cultures

Expt. No.	Skin Reactivity To 1 TU (E & 1 mm)	Cell Concentration /ml Culture	Transformation at 5 Days Mitotic Response %	SRF ASSAY Mean Diameter of Skin Reaction in mm: Stimulated Cultures	SRF ASSAY Mean Diameter of Skin Reaction in mm: Control Cultures
1	5 x 5	7.5×10^6	0	2 x 2 pallor	2 x 2 pallor
2	5 x 5	10×10^6	2.1	10 x 10 E & 1	-
3	10 x 10	25×10^6	0	9 x 4 pallor & I	-
4	2 x 2	25×10^6	0	5 x 5 pallor	7 x 7 pallor

ficant mitotic response in the PPD-stimulated lymphocyte cultures. In the remaining experiments the majority of lymphocytes were small, with a few blasts and one or two mitoses visible on scanning the entire slide. It was only supernatants from the stimulated cultures in experiment 2 that, in addition, produced an inflammatory reaction upon intradermal injection. Other supernatants induced only areas of pallor when assayed.

A further nine consecutive experiments carried out to attempt to define the cause for the failure of the lymphocyte cultures were similarly unsuccessful. In most instances, lymphocytes in these experiments remained small with nuclei often irregular and pyknotic or faint and poorly stained. Some larger faintly stained rounded nuclei might have been cells that had begun to transform and then had been affected by some toxic process. There was no evidence for infection in these cultures.

DISCUSSION

The results of this section, few in number, reinforce the contention that, in this system, transformation is a concomitant of the production of the inflammatory substances. However, it may not necessarily be the cells that eventually divide that produce the SRF and circumstances have already been discussed where soluble factors are produced in the absence of cell division (Spitler et al., 1970; Chaparas et al., 1970). Rather, cultures in which cells are actively dividing are presumably healthy ones where a lymphocyte response to antigen had occurred and where the soluble factors might be produced by a cell population that does not proceed to cell division. Similarly, in cultures in which transformation and mitosis does not occur and in which there is no evidence for SRF production, the cells may be incapable of responding to antigen. This failure could be due to some intrinsic defect in the cells themselves but more likely it is the effect of an extraneous influence upon the cells. Possible causes include infection with atypical organisms such as Mycoplasma, inhibitory sera or culture media and detrimental effects due to the presence of high doses of antigen or toxic chemicals such as preservatives.

There was no definite explanation found for the lack of significant transformation in the experiments discussed above, although a number of variables such as culture media and sera were examined. However, it did not appear profitable to continue with the rather intricate technique involved in setting up the Marbrook cultures for SRF production in these circumstances where one could not be sure that the culture conditions were adequate for cell survival and a specific antigen response. Rather, attention was concentrated upon the definition and elimination of these current problems of guinea-pig lymphocyte culture. This was carried out by examining the effects of such factors as culture media, sera and antigens upon the in vitro transformation of the lymphocytes and will be discussed in the following section.

In addition, in the Marbrook system, apart from technical difficulties involved in manipulating small volumes of cell suspension, antigen and serum, it was noted that changes occurred in the volume of culture medium in the inner chamber. These were probably due to differences in the osmotic and hydrostatic pressures between inner and outer chambers. But such changes, coupled with any movement of PPD that occurred, would modify the concentration of antigen to which the cells were exposed. If this was so, then the question arose as to how long an exposure to optimal concentrations of PPD was necessary to ensure the induction of maximal transformation. This problem was also examined and will be discussed in the next section.

IV. Factors affecting lymphocyte transformation *in vitro*.

The primary aim of these experiments, carried out over a period of approximately one year, was to produce a guinea-pig lymphocyte culture system in which consistent transformation in response to specific stimulation could be attained. The experiments were not designed primarily to investigate the effects of such variables as different batches of culture medium or serum upon the lymphocyte response but nevertheless a considerable amount of information regarding these factors was obtained. In the case of culture media, experiments were conducted using different batches of media until a batch that would support growth of the lymphocytes was found. At times cells failed to transform even in this medium and in these cases the cause was frequently found to be a particular sample of the serum used in the cultures. The initial experiments were carried out with PHA as the stimulant but, in addition, when the specific antigen PPD was used and difficulties were experienced in obtaining successful growth and cell division, the effects of various antigen preparations were examined, for in some of the cultures exposed to PPD it appeared that transformation had begun and then some toxic process had affected the cells. It was considered possible that a toxic effect, due to the continued presence of the antigen or of preservatives, in the form of phenol, in the diluent might have been the cause. If this were so, then a shorter duration of exposure to antigen might eliminate this effect. Thus the essential point to be determined was the length of time that the cells need to be exposed to antigen in order to ensure the induction of maximal transformation. This was the same potential problem that had occurred in the use of the Marbrook culture system, where changes in the inner chamber volume might have modified the dose of PPD to which the cells in the chamber were exposed.

MATERIALS AND METHODS

Animals: The animals used were male Hartley guinea-pigs weighing 500-1000 g.

Immunization: Where sensitised animals were used, these weighed from 500-700 g and were immunized with a suspension of 40 mg of freeze-dried BCG vaccine (Connaught) in 1 ml of saline and 0.5 ml CFA (BBL), 0.05 ml into each footpad.

Skin Tests: Skin reactivity was assessed as indicated in previous sections.

Preparation of Lymphocytes and Serum: Blood was withdrawn for the preparation of serum as well as for lymphocyte separation on 9% ficoll and 37.5% Hypaque as described in Section III.

Pooled guinea-pig serum was prepared in batches from healthy male guinea-pigs from the colony. All sera were heat-inactivated at 56° C for 30 min before use.

Culture Conditions:

Assessment of media effects: Mononuclear cells were cultured in 2 ml of culture medium with 20% pooled or autologous heat-inactivated guinea-pig serum, penicillin 100 units/ml, streptomycin 100 ug/ml and kanamycin 100 ug/ml. The culture media used included RPMI 1640 (GIBCO), Medium 199 Earles Base (GIBCO), Medium 199 Hanks Base (GIBCO), or Eagle's Minimal Essential Medium (GIBCO). Different batches of the same medium from the same source were also tested. PHA 2.5 ul/ml of culture or saline was added in 0.1 ml aliquots to cultures in triplicate. Cultures were incubated at 37° C in 100 x 13 mm sterile glass culture tubes, loosely capped in 5% CO_2 and air in an humidified atmosphere for 72 hr.

Assessment of sera effects: Mononuclear cells were cultured at a concentration of 1 x 10^6 cells/ml in 2 ml of Medium 199A containing 20% serum and antibiotics. In the initial group of experiments, cells were grown in autologous or pooled heat-inactivated guinea-pig serum and on one occasion in homologous serum stored from a previous experiment. PHA at a dose of 2.5 ul/ml of culture, or 0.1 ml saline was added to the tubes.

In the two final experiments, pairs of tuberculin-sensitive animals were examined, cells from each animal being cultured in Medium 199A in 20% serum, either autologous, in serum from the other member of the pair and in pooled guinea-pig serum. These cells were stimulated with PHA 2.5 ul/ml or PPD 2.5 ug/ml.

All cultures were incubated in triplicate in 100 x 13 mm sterile glass culture tubes, loosely capped, in 5% CO_2 and air in a humidified atmosphere at 37° C. PHA stimulated cultures were incubated for 72 hr and then harvested whilst PPD stimulated cultures were incubated for 120 hr.

Assessment of antigen effects: In the first experiment, mononuclear cells at a concentration of 1 x 10^6 cells/ml were exposed in 2 ml of RPMI 1640 to PPD 2.5 ug/ml (Connaught) for a period of time from 10-120 min at 37° C. The cells were washed once in cold medium and were resuspended in 100 x 13 mm culture tubes in 2 ml of RPMI 1640 containing 20% pooled guinea-pig serum and antibiotics. Additional cultures were also set up containing cells not previously exposed to PPD and antigen was added to tube cultures (at 2.5 ug/ml) and left in contact with the cells throughout the incubation period. The cultures, in triplicate, were incubated for 120 hr in 5% CO_2 and air at 37° C in an humidified atmosphere.

In subsequent experiments, the cells were initially exposed to 2.5 ug PPD/ml in culture medium containing 20% pooled or autologous guinea-pig serum for periods from 40-120 min and then were washed in cold medium RPMI 1640 before being resuspended once more in 2 ml of RPMI 1640 containing 20% pooled or autologous guinea-pig serum and antibiotics. These cultures were incubated in triplicate, along with identical cultures set up containing 2.5 ug PPD/ml, for 120 hr at 37° C in 5% CO_2 and air.

In two experiments, cells to be exposed continuously to antigen were incubated initially with the other cultures but without PPD and were then also washed before the addition of PPD to ensure that it was not the extra washes that affected the cell transformation. In addition, in two further experiments, cells to be exposed continuously to antigen were incubated with PPD for 40 min, washed, resuspended and a further addition of PPD was made. Neither of these two procedures modified the final results in any way.

Subsequently, it was possible to set up dose/response experiments comparing excipient-free PPD (Parke-Davis & Co., Montreal, P.Q., Canada) with PPD containing preservatives (Connaught Laboratories). Cells were cultured at 1 x 10^6 cells/ml in Medium 199 with 20% pooled guinea-pig serum and doses of PPD from 0.1-2.5 ug/ml. Cultures were harvested after 120 hr incubation.

Assessment of Stimulation: This was by 5 hr colchicine block using 0.2 ug/ml of culture and the assessment of the mitotic response was as described previously.

RESULTS

The influence of different batches of culture medium on the transformation of guinea-pig lymphocytes stimulated with PHA.

TABLE 8

Influence of Culture Medium on the Transformation of Guinea-pig Lymphocytes Stimulated by PHA

Expt. No.	Serum	Culture Medium	Batch	Mitotic Response (%)
1	PGPS	RPMI 1640	A	6.2
2	PGPS	RPMI 1640	B	0
		199 EBSS	A	3.3
		MEM	A	0
3	PGPS	199 EBSS	B	0.5
		199 EBSS	A	3.03
4	PGPS	199 EBSS	A	1.5
		199 HBSS	C	0.35
5	AGPS	199 EBSS	A	2.5
		199 EBSS	D	0
6	AGPS	199 HBSS	C	0
		199 HBSS	E	0
		199 EBSS	A	1.6
		199	9 parts D :1 part A	1.4

In general, lymphocytes in culture either responded well to PHA with transformation and cell division or the cultures consisted almost entirely of small pyknotic lymphocytes often in large clumps, with occasional larger faintly stained pink nuclei. Only those experiments in which inhibitory effects could be definitely ascribed to the culture medium are included. Where growth failed to occur in any of the media tested, these results are omitted since here the culture medium may not have been responsible.

There was a remarkable degree of consistency in that a medium that supported growth, e.g., 199A, regularly did so whilst others that did not, or did so very poorly, e.g., 199C, RPMI B, persistently failed.

The media were tested immediately upon arrival from the supplier. The exception to this was 199A, the most consistantly successful medium used, which had been stored for over a year before it was tested.

Supplements such as L-Glutamine or Non-Essential amino acids did not convert a poor culture medium into a good one. The only means by which such a conversion was accomplished was when nine parts of 199F, which did not support growth, was mixed with one part of 199A.

The influence of different batches of sera upon the transformation of guinea-pig lymphocytes stimulated with PHA or PPD.

In the experiments examining the effects of sera on transformation in culture, depicted in Table 9, ~~it~~ was found that there were marked differences in the ability of different batches of pooled sera to support growth. Thus, a good mitotic response occurred in two different experiments using PGPS(a) whereas there was no transformation in PGPS(b). In addition, cells at times failed to transform in autologous serum whereas a good mitotic response occurred in the pooled serum used (see experiments 2 and 3).

TABLE 9

Influence of Guinea-pig Serum on the Transformation of Guinea-pig Lymphocytes in Response to PHA 2.5 ul/ml

Expt. No.	Serum	Mitotic Response (%)
1	AGPS	0.13
2	HGPS (1)	0.06
	AGPS	0
	PGPS (a)	3.03
3	AGPS	0
	PGPS (a)	1.7
4	AGPS	2.1
	PGPS (b)	0
5	AGPS	0.43
	PGPS (b)	0

AGPS = autologous guinea-pig serum
HGPS = homologous guinea-pig serum
PGPS = pooled guinea-pig serum

Such inhibitory sera might modify the in vivo response to antigen, causing a loss of cutaneous reactivity and this was examined in the experiment depicted in Figure 17. In this experiment, two guinea-pigs were taken, one, guinea-pig A, was about four months old, had been sensitised to tuberculin and responded to 1 TU with a cutaneous reaction 8 x 10 mm erythema and induration. The other, B, was about 18 months old, had been sensitised over one year before, responding to 5 TU with a reaction 5 x 5 mm in diameter, but just prior to the experiment was judged tuberculin-negative on testing up to 250 TU. The cells and the sera of

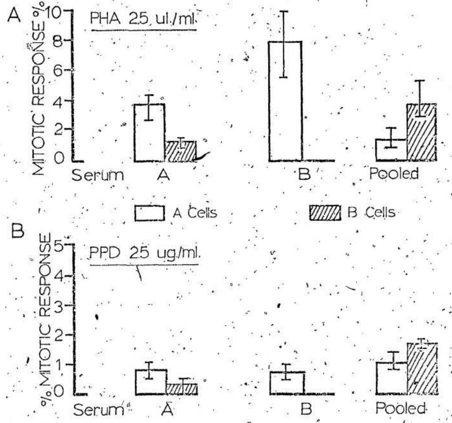


FIGURE 17. The mitotic response of peripheral blood lymphocytes from two guinea-pigs, A and B, stimulated with PHA (a) or PPD (b) and cultured in sera from either animal and in pooled guinea-pig serum. The range of results from triplicate cultures is shown. Plain histograms represent A cells and cross-hatched represent B cells.

Cutaneous Reactivities: A 1 TU → 8 x 10 mm E. & I.

B 250 TU → 0

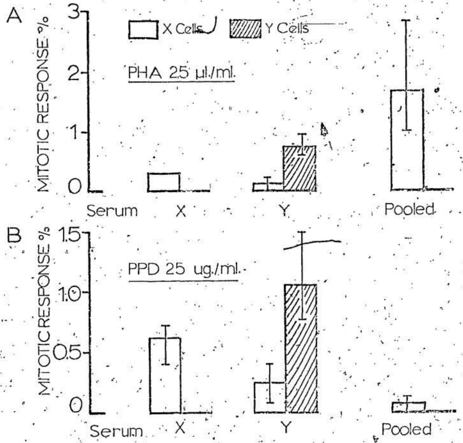


FIGURE 18. The mitotic response of peripheral blood lymphocytes from two guinea-pigs, X and Y, stimulated with PHA (a) or PPD (b) and cultured in sera from either animal and in pooled guinea-pig serum. The range of results from triplicate cultures is shown; plain histograms represent X cells and cross-hatches represent Y cells.

Cutaneous Reactivities: X 1 TU → 3 x 4 mm E. & I.

Y 1 TU → 0

5 TU → 5 x 5 mm E. & I.

these two animals in tissue culture produced the results indicated in Figure 17 on stimulation with PHA and PPD. These results indicate that Serum B is definitely inhibiting the response of B cells to PHA and PPD and that these cells are quite capable of responding to these stimulants in the other sera, both A and pooled. In addition, the inhibition is specific since cells from A are capable of responding to stimulation in B serum. Slides prepared from B cells incubated in B serum show scanty cellularity with only small pyknotic lymphocytes present, in complete contrast to the appearance of the cells in the stimulated cultures.

In a second experiment of this type, two guinea-pigs, both about 18 months old, were used, guinea-pig X reacting to 1 TU with cutaneous response 3 x 4 mm in diameter and Y reacting to 5 TU with a reaction 5 x 5 mm in diameter. The results from this experiment are depicted in Figure 18. In this experiment, a different pattern of inhibition is seen, when Y cells transform in autologous serum in response to PHA and PPD but do not grow in serum from X nor in pooled serum. However, X cells grow in all three types of sera, but less well in Y serum than in autologous serum. The pooled serum is the same batch as that used in the previous experiment and the fact that Y cells will not grow in this serum, when A, B and X cells will, once more emphasizes the complexities of the serum effects in this culture system.

<u>The effects upon sensitised guinea-pig lymphocytes of varying the duration of antigen exposure, using two different preparations of PPD.</u>

The results of the first experimental investigation into the effects of a variable antigen exposure time (Figure 19) demonstrated a definite fall in mitotic response with increasing exposure time. The

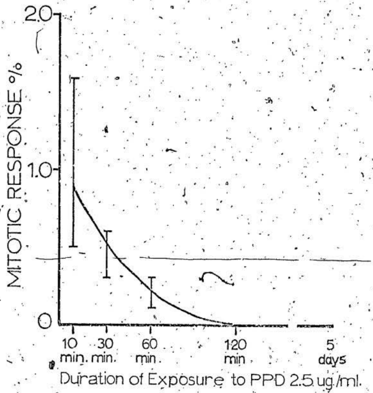


FIGURE 19. The fall in the mitotic response of sensitised guinea-pig lymphocytes to PPD at 5 days, with duration of antigen exposure in serum-free medium. The range of results in triplicate cultures is shown.

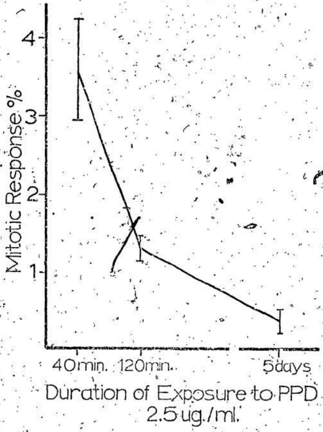


FIGURE 20. The fall in the mitotic response of guinea-pig lymphocytes to PPD with duration of antigen exposure in serum-containing medium. The standard error of the results obtained is shown.

levels of mitotic response were, however, rather low, being mostly less than 1.0%. If the results obtained were due to antigen toxicity, then this toxicity would be more profound in serum-free medium, because of the lack of antigen-binding by plasma proteins and possibly because of more rapid cell death in serum-free medium at 37° C. For this reason, the experiment was repeated in two further experiments with the antigen exposure being carried out in 20% serum (Figure 20). Here the same fall in mitotic response with increased duration of exposure to antigen was seen, except that the mean values for the mitotic response were now considerably higher and differed significantly from each other as indicated in Table 10.

TABLE 10

Differences in Mitotic Response at 5 Days after Variable Exposure Time to PPD 2.5 ug/ml. Results are for 9 cultures in each group.

Duration of Antigen Exposure	Mean Mitotic Response % ± S.D.	t Statistic	
40 min	3.5 ± 1.6	$p < 0.01$	$p = 0.001$
120 min	1.3 ± 0.4		
5 days	0.4 ± 0.4	$p < 0.05$	

Thus the mitotic response after 40 min exposure to PPD is significantly different ($p = 0.001$) from the response obtained after continuous exposure to the antigen, and is also significantly different from the response following 120 min exposure ($p < 0.01$). The difference between 120 min exposure and continuous exposure is significant at the 5% level.

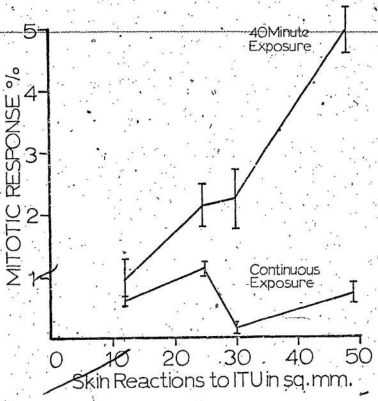


FIGURE 21. The relationship between the cutaneous reactivity of sensitised guinea-pigs to tuberculin and the in vitro response of their lymphocytes to PPD, after 40 min and continuous exposure to the antigen. The range of results in triplicate cultures is shown.

In four experiments using guinea-pigs of varying cutaneous skin reactivity to tuberculin, the mitotic response at 5 days was measured in lymphocyte cultures exposed to PPD 2.5 ug/ml culture for 40 min and continuously. When skin reactivity was plotted graphically against the mitotic response it was found that a straight line relationship existed between the response after 40 min exposure to antigen and cutaneous sensitivity (Figure 21). However, such a correlation did not apply for transformation after continuous antigen exposure.

The possibility was raised that these results might be due to preservatives contained in the PPD preparation used in these experiments, i.e., phenol 0.3%, rather than a direct toxic effect from the antigen itself. To test this, excipient-free PPD (Parke-Davis) was compared with preservative containing-PPD (Connaught) in the form of dose-response experiments (Figure 22).

Whereas a linear relationship existed between the mitotic response and concentration of excipient-free PPD, the dose/response curve for preservative containing antigen appeared flattened at the higher doses, which could be the result of increasing amounts of preservative in the cultures. This flattening was seen in dose/response experiments using three different batches of PPD (Connaught), whereas excipient-free PPD gave an approximately linear curve.

However, in variable antigen exposure time experiments using the preservative-free preparation, it was found that a 40 min exposure to antigen still resulted in an increased mitotic response in comparison with continuous exposure, but the difference was now only significant at the 10% level (Table 11).

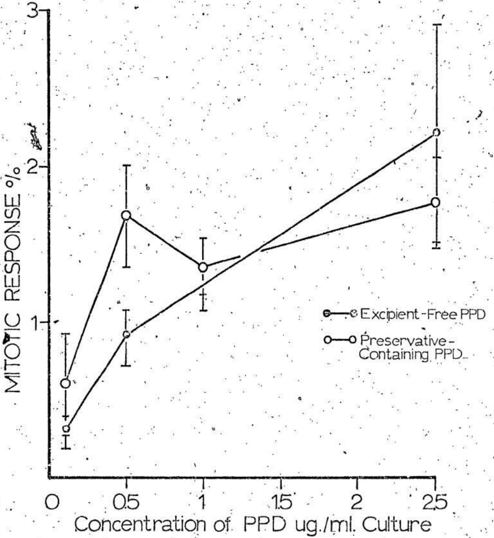


FIGURE 22. The effect of increasing concentrations of preservative-containing and preservative-free PPD on the mitotic response of sensitised guinea-pig lymphocytes. The standard error of the results is shown.

TABLE 11

Differences in Mitotic Response at 5 Days after a Variable Exposure Time to PPD 2.5 ug/ml Free from Preservatives Results are for 9 cultures in each group.

Duration of Antigen Exposure	Mean Mitotic Response % ± S.D.	t Statistic
40 min	1.15 ± 0.83	$p = 0.1$
5 days	0.59 ± 0.48	

DISCUSSION

It may not be unexpected to find that a certain type of culture medium supports the growth of a particular cell type better than other media. But it does seem to be unusual when one batch of medium is significantly better than other apparently identical batches of the same medium. This is assuming that the batches of chemically defined media, commercially produced, contain identical constituents. If so, then, in the case of Medium 199A (Table 8) which, previously unopened and approxmately one year old, sustained the growth of guinea-pig lymphocytes on most occasions, the possibility arises that some advantageous change occurred in the medium during storage at 4° C. Since there are 62 constituents that could, in theory, have changed, an attempt to define the precise difference is unlikely to succeed and is certainly beyond the scope of this thesis. Obviously, some factors are more prone to alteration than others, e.g., L-Glutamine, ascorbic acid, L-cysteine, L-methionine, d-Biotin, but it is difficult to see what advantage any such alterations would be. Since human lymphocytes were grown in some of the media that guinea-pig lymphocytes could not be grown in, these media were obviously capable of supporting normal growth and division but could not supply an additional requirement of the guinea-pig cells. Both human and guinea-pig cells require ascorbic acid for normal growth and development and it has been shown that scorbutic tuberculin-sensitive guinea-pigs fail to react on skin test (Mueller & Kies, 1962). But lymphocytes from these animals transform normally <u>in vitro</u> in response in PPD (Zweiman et al., 1966), although these experiments were carried out in TC-199 which contains ascorbic acid. Nevertheless, it is unlikely that vitamin C deficiency is the cause for differences in growth in various batches of Medium 199, since supplements of ascorbic acid to these media did not affect the outcome of the cultures. Whatever the nature of the requirement for growth of guinea-pig lymphocytes, it is obviously not supplied by the sera used in these experiments. The balance between success and failure in this culture system must be exquisitely sensitive if it responds to only minor changes in the constituents of the culture medium. The fact that Medium 199A may be diluted ten times and still maintain growth would appear to support this suggestion.

Perhaps the essential defect lies in the sera, deficient in some essential material supplied by Medium 199A, possibly in the form of small molecules such as vitamins or coenzymes, or essential aminoacids. If so, then most of the sera examined were so deficient.

Ling (1968) has suggested that there are five factors which may be considered in the analysis of the effects of a particular serum.

(a) Macromolecules such as α-globulins or acid mucopolysaccharides which are protective or promote growth in some ill-defined manner (Morrison et al., 1965; Michl & Svobodova, 1966; Tozer & Pirt, 1964).

(b) Small molecules, e.g., nucleosides, vitamins, hormones, coenzymes which supply essential trace nutrients not present in the medium (Ambrose & Coons, 1963; Coulson & Chalmers, 1967).

(c) Factors, possibly antibodies, which neutralize or combine with the stimulant (Heilman & McFarland, 1966).

(d) 'Natural' antibodies to antigenic sites on the surface of the lymphocytes, which may be stimulatory or cytotoxic.

(e) Foreign antigens, especially applicable where heterologous serum is used.

In the present work, many of the guinea-pig sera tested were incapable of supporting growth. Such defective sera have been described previously by Phillips and Zweiman (1970) who noted that sera from some animals was associated with little or no proliferative response of the cells to PPD and that considerable variation was noted between lots of pooled normal guinea-pig serum. It was generally observed that if the serum from an individual animal was associated with a good *in vitro* proliferative response, then this good response was seen both in autologous and homologous cell populations. A similar relationship was seen in some sera tested here, where cells grew well in one batch of pooled serum (Table 9) or in sera from a particular animal. A possible cause for these variations could be non-specific macromolecules such as α-globulins or acid mucopolysaccharides described by such workers as Morrison et al. (1965) and Michl and Svobodova (1966). A second possible explanation might be the presence of factors neutralizing or combining with the PPD as described by Heilman and McFarland (1966). Certainly antibodies to PPD can be detected in guinea-pigs sensitised with BCG and CFA (Wasserman et al., 1969) and such antibodies could, in a detrimental way, interfere with the interaction of sensitised cells with the antigen, either directly or in the processing of antigen by monocytes or macrophages. But, in addition, one could also visualize an advantageous effect of such antibodies in the situation where the presence of large amounts of free antigen was itself damaging to the cells. Phillips and Zweiman (1970) noted a depression of isotope incorporation into DNA in stimulated cultures of guinea-pig lymphocytes at levels of antigen equivalent to 5 ug PPD/ml of culture and this depression fell below control levels at 20 ug/ml. Interestingly, Schrek (1963) has described an inhibition in the development of macrophages from monocytes in cultures of human peripheral blood cells at levels of PPD from 5-20 ug/ml. Monocytes persisted as rounded-up cells, with peripherally placed nuclei, crescentic in shape and without nucleoli. Certainly, the importance of monocytes and macrophages in the response of sensitised lymphocytes to antigen has been amply demonstrated (Oppenheim et al., 1966; Heilman & McFarland, 1967; Hersh & Harris, 1967; Levanthal & Oppenheim, 1967; McFarland, 1967; Seiger & Oppenheim, 1970). However, whether the depressing effect of high doses of PPD is exerted upon monocytes or lymphocytes, the presence of a certain amount of neutralizing antibody could conceivably enhance the degree of transformation found in the cultures by reducing the concentration of free antigen available. This point will be returned to in the consideration of the effects of varying the duration of antigen exposure.

The specific inhibition of autologous lymphocyte transformation seen in the experiment comparing old and young guinea-pigs (Figure 17) cannot be explained on the basis of general inhibitory or stimulatory factors being present in the sera, unless cells from the old animal had a special requirement for such factors. The simplest explanation would be the presence in the serum of antibodies reacting against self-antigens upon the lymphocytes of the old guinea-pig. This would explain the specificity of the serum effect but not its nature nor its cause. Certainly, such antibodies would have to prevent interaction of the mononuclear cells with the antigen without causing destruction of the cells, since reactive cells were present and capable of responding in a suitable environment, the serum from the younger animal. Since the PHA response is lost, presumably thymus-derived cells are affected, and a further possibility is of a relative deficiency of some substance necessary for thymic-dependent cells to react to PHA or PPD, perhaps a thymic factor. Cells from the young animal were able to react in the inhibitory serum, but they could have conceivably carried sufficient of the material with them to maintain the in vitro response, whereas cells from the old guinea-pig may have been chronically deficient in the substance. It was to exclude such a serum deficiency that the second experiment in this group was carried out, comparing two aged guinea-pigs (Figure 18). These animals had not completely lost their skin reactivity to antigen, in fact, none of the remaining animals in the colony had, but were of comparable age to the first animal tested. However, it was found that cells from the most reactive guinea-pig, as regards tuberculin testing, were able to react in all the sera tested, whereas the less reactive animal responded only in its own serum. These results are not compatible with either one of the two hypotheses suggested to explain the first experiment and indicate the complexity of serum effects in tissue culture. It should be noted, however, that the inhibition in this experiment was consistent both for PHA and PPD responses, as it was in the first experiment. More perplexing was the failure of Y cells to grow in the pooled serum in which cells from the other animals tested were capable of growth.

Although only a single experiment, the interesting difference between young and old guinea-pigs is worthy of comment, since it indicates a field of study that should, perhaps, be pursued later in more detail. The inhibitory nature of the serum from the old animals to its own cells may have relevance to the ageing process in general. The depression of lymphocyte reactivity to PPD or PHA with increasing age has been described in the past (Pisciotta et al., 1967; Dequeker et al., 1968, Nillson, 1971) and it is possible that this depression could be ascribed to changes occurring in the thymus gland (Walford, 1969; Burnet, 1970). Certainly, the importance of the thymus gland in the adult animal has been well documented (Metcalf, 1965; Miller, 1965). Such an ageing process could result in the depression of thymic production of some essential factor, in the modification of antigens on lymphocytes causing them to be recognized as not-self, or in the development of mutated clones of immunocompetent cells reacting against self-antigens. The relevance of these considerations to the current experiments concerns the relationship of the negative or weakly positive tuberculin reaction in aged animals (and in weakly reactive animals or man in general) to the <u>in vitro</u> activity of immunocompetent cells. Is the failure of these cells to grow satisfactorily in culture in autologous serum an explanation for the failure of the animal to mount a significant skin reaction to tuberculin?

The fact that sensitised lymphocytes require only a short exposure time in order to respond to PPD has been demonstrated before by Caron

(1967) using human peripheral blood lymphocytes. In his experiments, the degree of transformation and the mitotic response induced after exposure period of from 10 sec to 5 min was greater than after continuous exposure (M.R. 1.4% as compared to 0.4%) but the significance of this difference cannot be ascertained from the results. Indeed, the difference was not commented on. The type of PPD used by Caron was not mentioned, so the possible role of preservatives in this effect cannot be determined. Even so, it would appear, from the results of Caron and the results of experiments reported here (Table 10) that the degree of transformation following a short exposure of sensitised mononuclear cells to PPD is equally as good as that occurring with continuous exposure to the antigen. Indeed, the evidence would indicate that it is actually better and it is suggested that, in fact, this advantage of short antigen exposure time is the result of preservatives contained in the PPD. Thus the dose/response curve using preservative-containing antigen is flattened at the higher doses (Figure 21) and the enhancement of the mitotic response with short duration of exposure is greater when PPD containing preservative is used (Tables 10 and 11). However, there still remains the possibility that there is, in addition, a detrimental effect of the continuous presence of the antigen itself that reduced the mitotic response below the optimum, since even when using excipient-free PPD there was a greater mitotic response with a short duration of exposure to the antigen (Table 11). The single experiment (Figure 19) where pre-exposure of the cells to PPD in serum-free medium was carried out might be considered to support this suggestion, for here mitotic responses were low and fell to zero after only 120 min exposure to the antigen. However, this might have been an effect solely of the preservative. Thus a shortened exposure time might act by reducing the duration of cell exposure to the toxic effects of PPD whilst ensuring that lymphocyte receptors or monocytes are sufficiently well primed with antigen. In the presence of serum containing antibodies to PPD, it might be that sufficient unbound antigen is present to stimulate transformation while excess antigen is bound to specific antibody present in the serum. Complete inhibition of transformation might occur when no free antigen is available to stimulate the cells. However, the toxic effect itself could be exerted upon sensitised lymphocytes, monocytes or macrophages and a distinction with regard to this cannot be made from the data presented. In experiments by Seeger and Oppenheim (1970), it was found that pre-incubation of macrophages with 20 ug PPD/ml for periods from 1/2-6 hr before washing and adding the cells of sensitised guinea-pig lymphocytes resulted in a reduction in transformation as compared to lymphocytes and macrophages incubated continuously with the antigen. In addition, the optimal period of exposure of short duration was 4 hr. These were macrophages from peritoneal exudates and not peripheral blood monocytes and so these results may not be directly comparable with the results of Schrek (1963). However, it does suggest that protecting the lymphocytes from initial exposure to the antigen does not enhance their transformation. In addition, the results might indicate that macrophages are adversely affected by the antigen exposure. However, in this case it would be expected that the optimal response would be obtained following the shortest period of exposure, i.e., 30 min, provided that that was sufficient time for the macrophages to take up or react with the antigen. The fact that in the present experiments (Table 10) only 40 min exposure was required might indicate that the response, in fact, proceeds more efficiently when the cells are presented together with the antigen. The results of Seeger and Oppenheim (1970) presented above would appear to support this.

Additional in vivo evidence that with continuous antigen exposure in the system used here one is seeing lymphocytes responding in a sub-optimal way to antigen comes from the demonstration of an approximately linear relationship between the cutaneous hypersensitivity of the donor animal and the mitotic response after a short exposure time (Figure 21).

Although time-consuming and even frustrating, experimental analysis of tissue culture conditions, especially serum and antigen effects, would appear to be necessary in order to attain the familiarity required for comparing quantitatively the in vitro responses of immunocompetent cells. There is obviously scope for continued work here, particularly with regard to the dissociation of lymphocyte and monocyte contributions to the total in vitro response and the effects of serum and antigen upon these cell types.

V. The investigation of inflammatory factors produced by guinea-pig peripheral blood lymphocytes stimulated with PPD.

A number of investigators of the putative soluble mediators of delayed hypersensitivity have produced the active supernatants by the incubation of sensitive guinea-pig lymphocytes, derived from lymph nodes, in serum-free medium, subsequently concentrating these supernatants by vacuum dialysis or lyophilization (Bennett & Bloom, 1968; Heise & Weiser, 1969; Remold et al., 1970; Bernstein et al., 1971). However, in the development of a proposed animal model for subsequent human experiments, it would obviously be more sensible to use mononuclear cells derived from peripheral blood. The use of human peripheral blood lymphocytes for the production of MIF was described by Rocklin et al. (1970), again utilising a serum-free culture system. In this case, the active supernatants were subsequently assayed using guinea-pig peritoneal exudate cells as originally described by David et al. (1964).

In this final series of experiments using guinea-pig peripheral blood lymphocytes, separated on ficoll/Hypaque, inflammatory supernatants were produced by culture in serum-free medium with PPD for 3 days, subsequently concentrating the supernatants after dialysis, by lyophilization. Lymphocyte transformation in response to antigen was assessed, in 20% autologous serum, at 5 days by colchicine block, since cell division was found not to occur in cultures lacking serum. This effect of the removal of serum from lymphocyte cultures has been noted previously (Ling, 1968; Walker & Lucas, 1969). As a further assessment of the in vitro response to antigen, serum-free supernatants were also assayed for the presence of MIF, by a modification of the technique described originally by David et al. (1964).

It was originally hoped that it might be possible to correlate SRF production in vitro with cutaneous hypersensitivity to PPD in vivo in a quantitative as well as qualitative manner. One problem that might arise using the present system is that the immune response in serum-free medium may not be found to correlate quantitatively with the cutaneous reactivity of the donor in the way that transformation in the presence of serum, for example, has been shown to correlate (Mills, 1966; Oppenheim, 1968). Thus, the occurrence of inhibitory factors in autologous serum might modify the immune response as discussed in Section IV. In addition, the dependence of lymphocyte reactivity on serum factors important in the maintenance of cell viability and general cell functions such as protein or RNA synthesis might seriously affect quantitatively the production of mediators. Thus, in experiments in guinea-pigs or in man, the failure to demonstrate SRF by intradermal injection of serum-free culture supernatants could be the result of a low level of production of the mediator. However, such negative findings might be interpreted as indicating that SRF does not exist or, in the case of human experiments, that species differences preclude its demonstration in the guinea-pig. Indeed, in this context, the fact that MIF has been shown to be produced in serum-free cultures (Bennett & Bloom, 1968; Remold et al, 1970; Rocklin et al., 1970) is one reason for including in the experimental design this additional in vitro assessment of mediator production which is also an indicator of a lymphocyte response in the cultures.

MATERIALS AND METHODS

Animals: Male Hartley strain guinea-pigs weighing 500-1000 g were used in these experiments.

Immunization: Guinea-pigs weighing 500-700 g were immunized once with a suspension of 40 mg of freeze-dried BCG vaccine (Connaught Laboratories) in 1 ml of saline and 0.5 ml CFA (BBL), 0.05 ml of the suspension being injected into each footpad. Control normal guinea-pigs received no immunization.

Skin Tests: Skin reactivity was assessed, approximately 4 weeks after immunization, by intracutaneous injection of PPD, 1 TU, 5 TU or 250 TU in 0.1 ml (Connaught Laboratories) into the shaved abdominal skin. Reactions were assessed at 24 hr by measurement of the diameter of erythema and induration.

Preparation of Lymphocytes: The animal was exsanguinated by intracardiac puncture with an 18 G 1½ in disposable needle, whilst under ether anaesthesia, using sterile technique. The blood was withdrawn into a plain 30 ml plastic disposable syringe (Jelco Laboratories) and after exsanguination was complete, 8-10 ml of the blood was transferred to a sterile glass culture tube and left to clot. Immediately following the transfer, 2.7% EDTA, pH 7.4 was added to the syringe (1 ml of anticoagulant to 20 ml of blood) and was mixed thoroughly with the blood. Serum for heat activation at 56° C for 30 min was prepared from the clotted blood as described previously. Anticoagulated blood was diluted 1:1 with isotonic saline and was layered carefully onto 10 ml of a mixture of 9% ficoll and 37.5% Hypaque, in 50 ml sterile glass round bottomed centrifuge tubes. The tubes were then centrifuged at 400 g at the interface for 40 min at room temperature. At the end of that time, the clear plasma/saline level was pipetted off and discarded and the mononuclear cell fraction at the interface was completely removed using a Pasteur pipette. This was transferred to a cold, conical bottomed sterile plastic centrifuge tube and was diluted up to about 40 ml with cold HBSS containing 10 units of sodium heparin per ml. The diluted mononuclear cell fraction was then centrifuged at 500 g for 16 min at 4^{O} C. After discarding the cell-free supernatant, the cell button was resuspended in a known volume (usually 10 ml) of HBSS containing heparin. A cell viability by trypan blue exclusion and a cell count using a haemocytometer was then performed. A drop of the cell suspension was smeared upon a serum-coated slide for later fixation with methanol and staining with Giemsa for a differential count. In addition, a drop or two of the cell suspension was incubated for 30 min at 37^{O} C with latex particles and heat-inactivated foetal calf serum. At the end of the incubation period, the cells were resuspended and introduced into a small slide chamber prepared by waxing a square coverglass onto a microscope slide. After sealing, the cells were incubated in the slide chamber for about 10 min, with the coverglass downwards, and were then examined by phase-contrast using a Wild Inverted M40 microscope. Cells were assessed with regard to their shape and size, presence or absence of latex particles, nuclear and cytoplasmic characteristics and method of movement. A differential count was performed, scanning the slide chamber once from edge to edge. The total number of cells counted was usually 500-1000.

Culture Conditions: For the preparation of SRF, mononuclear cells were incubated, at concentrations of 1 - 3.8 x 10^{6} cells/ml in plastic 25 ml culture flasks (Falcon Plastics) in a total volume of 8-10 ml of Medium 199 (GIBCO) containing penicillin 100 u/ml, streptomycin 100 ug/ml and kanamycin 100 ug/ml (GIBCO). Excipient-free PPD (Parke-Davis) in Medium 199 (GIBCO) buffered with Hepes (25 mM N'-2-Hydroxy-ethylpiperazine-N'-Ethanesulfonic Acid) was added to stimulated cultures to give a final concentration of 2.5 ug/ml whilst an equal volume (0.1 ml) of the medium was added to control cultures. The flasks were incubated for 3 days at 37° C in an humidified atmosphere of 5% CO_2 and air.

For the assessment of transformation, mononuclear cells were incubated, at a concentration of 1 x 10^6 cells/ml in 2 ml of Medium 199 containing 20% heat-inactivated autologous serum and antibiotics. Excipient-free PPD 5 ug in 0.1 ml was added to stimulated cultures and 0.1 ml of medium was added to controls. Cultures were incubated, in triplicate, in 100 x 13 mm sterile glass culture tubes for 5 days at 37° C in an humidified atmosphere of 5% CO_2 and air.

Preparation of SRF: Supernatants were withdrawn carefully from the flasks at 3 days and were centrifuged at 16,000 g for 20 min. The cells from the flasks, resuspended in a few drops of medium, were smeared on serum-coated slides, fixed and subsequently stained with Giemsa. Supernatants were pipetted off from the deposit after centrifugation and the appropriate amount of PPD was added to the control supernatants to give an equivalent concentration to the starting concentration of PPD in the stimulated cultures. The supernatants were then dialysed, using 1 23/64" dialyzer tubing double-knotted at each end, against 0.15 M sodium chloride for 24 hr and against distilled water for 24 hr. Dialysis was carried out at 4° C in an Oxford Multiple Dialyser (Fisher Scientific Co. Ltd.). At the end of the dialysis period, the dialysed supernatants were filtered through a cellulose filter of pore size 0.22 u (Millipore Ltd.). A glass fibre prefilter (Millipore Ltd.) superimposed directly on the bacterial filter in a single filter holder increased the ease of filtration. Supernatants were lyophilized, in 8-10 ml aliquots, and the lyophilized material was stored at -20° C until used.

Assessment of Transformation: Transformation in the tube cultures was assessed at 5 days by the use of colchicine 0.2 ug/ml of culture to block mitosis for 5 hr. At the end of that period, the cells were prepared by the technique already described in detail in Section I. The Mitotic Response (MR) was then determined by counting the number of mitoses present in 1000 mononuclear cells. This was expressed as a percentage in the final results.

SRF Assay: The lyophilized supernatants were taken and dissolved carefully in 0.2 ml of Medium 199 (Hepes buffered) containing antibiotics. The supernatants (concentrated 40-50 X) were drawn carefully into 1 ml tuberculin syringes through a 20 G needle, subsequently exchanged for a 30 G ½" disposable needle. A volume of 0.05 ml of each supernatant tested was injected intradermally into the shaved abdominal skin of normal guinea-pigs. Reactions were observed for up to 24 hr but were measured at their peak at 4-6 hr by the diameter of erythema and induration present, the degree of erythema and the increase in double skin thickness, measured using the Schnelltaster (System Kröplin, Type A.02T, H.C. Kröplin, Schlüchtern, Hessen, Germany). The remaining concentrated supernatant was diluted up to a total volume of 0.7 ml (concentrated now 5-6 X) with Medium 199 supplemented with 20% foetal calf serum for use in the MIF Assay.

Histology: Biopsies were taken from the skin reactions at 6 hr, were fixed in formaldehyde fixative and stained with hematoxylin and eosin.

MIF Assay: Peritoneal exudate cells from normal guinea-pigs were induced by the intraperitoneal (i.p.) injection of 10 ml of 2.5% starch gel in saline (Starch-hydrolysed, Connaught Laboratories). After 3 days, the animal was anaesthetised with ether and 30-40 ml of HBSS containing sodium heparin 10 u/ml was injected i.p. The abdomen was kneaded gently for 10 min and then a sterile plastic catheter (Bardic Around Needle Catheter, 14 Ga, 0.058 I.D., 2¼" long; C.R. Bard, Inc., Murray Hill, N.J., U.S.A.) was inserted into the peritoneal cavity and the fluid was drained from the abdomen into a cold plastic 25 ml culture flask (Falcon Plastics). The peritoneal exudate cells were washed twice in cold HBSS containing 10 units of sodium heparin per ml, spinning each time at 250 g for 5 min at 4° C. After resuspending the cells in a known volume of HBSS (usually 10 ml) a cell count was performed using a haemocytometer and viability was assessed by trypan blue exclusion. This was found to be greater than 90% on most occasions (mean 93% ± 7). A differential count was carried out using phase-contrast microscopy after incubation of the exudate cells with latex and heat-inactivated foetal calf serum. Macrophages were usually found to be well spread, having phagocytosed large numbers of the latex particles. In the series of experiments carried out, the mean differential counts were: granulocytes 31% ± 16, lymphocytes 15.5% ± 7, macrophages 54% ± 17. The cells were finally resuspended, at a concentration of 40 x 10^6 cells/ml in Hepes-buffered Medium 199 containing 20% heat-inactivated foetal calf serum and penicillin 100 units/ml, streptomycin 100 ug/ml and kanamycin

100 ug/ml. Capillary tubes (Non-heparinized Micro-Hematocrit, I.D. 1.1-1.2 mm, Fisherbrand, Fisher Scientific Co. Ltd.) were filled with the cell suspension and sealed at one end with either paraffin wax (Histowax, M.P. 54-56^{o} C, Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A.) or with vinyl plastic putty (Critoseal, Sherwood Medical Industries Inc., St. Louis, Missouri, U.S.A.) and were centrifuged at 130 g for 2 min in a clinical centrifuge. The tubes were cut at the interface and the portion containing the cells was placed in a Mackaness-type chamber (Disposable 'Lexy' Culture Chamber, Mini-Lab, Durvernay, Laval, Quebec, Canada), two capillaries in each chamber, held in place with silicone vacuum grease (Dow Corning Corporation, Midland, Michigan, U.S.A.). A sterile coverslip was sealed onto the top of each chamber with paraffin wax and the chambers were filled with the concentrated supernatants to be assayed. They were incubated at 37^{o} C for 24 hr and the area of migration was projected, using a Zeiss Projecting Microscope (Carl Zeiss, Germany) onto a sheet of stiff paper, 0.4 mm thick, and was then outlined. The outline of the area of migration was subsequently cut out and weighed. The results were expressed as:

$$\% \text{ Migration} = \frac{X}{Y} \times 100$$

where X = the weight of the area of migration in supernatants from stimulated cultures

Y = the weight of the area of migration in supernatants from unstimulated cultures.

The reproducibility of the preparative technique was assessed by measurement of the migration from twelve capillary tubes contained in five different Mackaness chambers filled with the same test medium. The standard deviation of the results obtained was ± 16% and the standard

TABLE 12

The Production of SRF and MIF by Sensitised Guinea-pig Lymphocytes Stimulated with PPD

Expt. No.	Skin Reactivity of Donor Animal in mm Erythema and Induration	Cell Concn. x 10^6/ml	Antigen Concn. ug/ml	Trans-formn. in autologous Serum	SRF ASSAY			MIF ASSAY	
					Suprnt. Concn.	Inflammatory Response in mm E & I (Increase in % Skin Thickness) Stimulated Supernatants	Unstimulated Supernatants	Suprnt. Concn.	% Migratn.
1	1 TU → 3 x 4	2.5	2.5	0.56	x 50	5 x 5 (76%)	-	x 6	71
2	1 TU → 5 x 5	1.0	2.5	1.4	x 40	15 x 11 (44%)	-	x 5	47
3	1 TU → 3 x 5	1.0	2.5	0.13	x 40	10 x 10 (20%)	-	x 5	57
4	1 TU → 3 x 4	3.0	2.5	0.83	x 20	10 x 7 (42%)	7 x 7 I(25%)	x 2.5	71
5	1 TU → 5 x 6	1.0	2.5	-	x 40	-	-	x 5	104
6	5 TU → 8 x 10	2.5	2.5	-	x 50	-	-	x 6	113
7	5 TU → 7 x 8	3.0	10	-	x 50	-	-	x 6	94
8	5 TU → 10 x 10	2.25	10	0.03	x 40	6 x 6 (20%)	3 x 3 (0)	x 5	116
9	5 TU → 8 x 7	3.8	10	3.8	x 40	12 x 14 (43%)	-	x 5	77

error of the mean for 12 capillary tubes was ± [illegible].

RESULTS

<u>The production of SRF and MIF by sensitised guinea-pig lymphocytes stimulated with PPD.</u>

In the results shown in Table 12, it can be seen that supernatants from stimulated lymphocyte cultures had inflammatory properties (mean reaction 95 ± S.D. 63 sq mm) whereas those from unstimulated cultures in most instances did not (mean reaction 6 ± 16 sq mm). This difference was significant (t test) at $p < 0.005$. On the two occasions (experiments 4 and 8) where reactions occurred with supernatants from unstimulated cultures, these reactions lacked either erythema or induration. Considering that the supernatants injected had been concentrated 20-50 times, the lack of non-specific reactivity was quite surprising. Active supernatants produced reactions characterised by erythema and induration, although the skin thickening did not have the firmness of the typical tuberculin response and must have had a significant element of oedema. The erythema was never very prominent (Figure 23) but could still be measured. These reactions reached a peak at from 4-8 hr and were usually measured at 6 hr. By 24 hr little remained of the responses except for a small pale nodule at the site of the injection. The presence of inhibition of the migration of peritoneal exudate cells in the MIF assay coincided with the presence of SRF in the supernatants in all but one experiment.

With regard to the correlation of the production of soluble factors with transformation, it can be seen that only where transformation and cell division occurred in the serum containing cultures, was

FIGURE 23. The inflammatory response produced by the injection of supernatants from stimulated guinea-pig lymphocyte cultures is shown at X and Y and the lack of inflammation at the site of injection of the control (unstimulated culture) supernatant is visible at the bottom of the photograph. This reaction was at 6 hours after intradermal injection into a normal unimmunized guinea-pig.

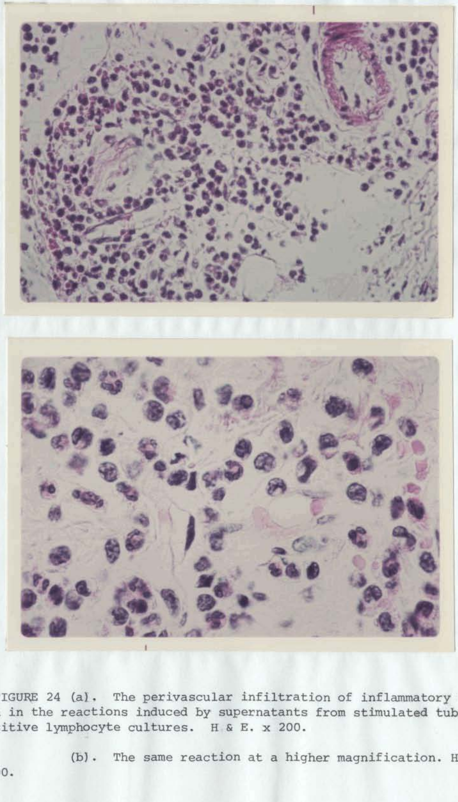


FIGURE 24 (a). The perivascular infiltration of inflammatory cells seen in the reactions induced by supernatants from stimulated tuberculin-sensitive lymphocyte cultures. H & E. x 200.

(b). The same reaction at a higher magnification. H & E. x 800.

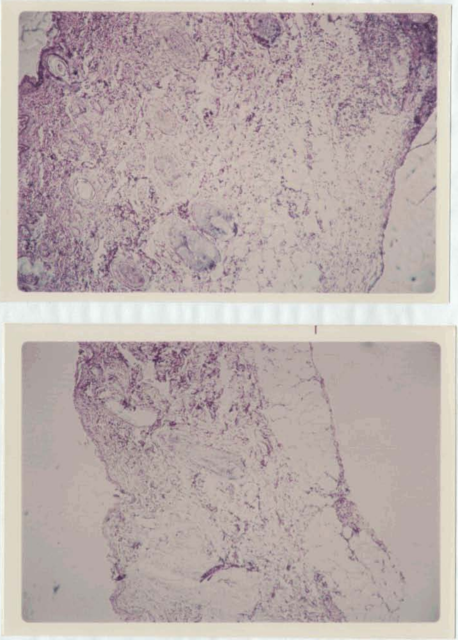


FIGURE 25. The histology of the reaction induced upon intradermal injection into a normal guinea-pig of supernatant from stimulated (a) and unstimulated (b) cultures as it appeared at 6 hours. H & E. x 32.

SRF and MIF detectable in the serum-free supernatants. In addition, it appeared that larger skin reactions were induced by the supernatants from experiments with the higher mitotic responses, although a definite relationship between cutaneous reactivity to tuberculin and the mitotic response was not apparent. However, mitoses and transformed cells were not visible in smears prepared from serum-free cultures, even when soluble factors had been produced. Lymphocytes were small, with pyknotic and irregular nuclei, whilst larger cells had degenerated beyond recognition and by trypan blue exclusion most of the cells (98 ± 1.3%) were dead.

Microscopically, the six hour skin reaction induced by supernatants from stimulated cultures was characterised by a mixed polymorphonuclear-mononuclear infiltrate in the deeper dermis, mostly around vessels between the dermis and adipose tissue (Figure 24 a & b). There was some infiltration of cells also into more superficial areas to just below the epidermis. Supernatants from unstimulated cultures produced a slight infiltration with a few mononuclear cells and polymorphs, spread evenly throughout the dermis without the predominantly perivascular infiltration in the deep dermis (Figure 25 a & b).

DISCUSSION

Using lymphocytes separated by centrifugation on ficoll-Hypaque from the peripheral blood of tuberculin-sensitive guinea-pigs, it is possible to demonstrate, in supernatants from cultures of these cells stimulated with specific antigen, a substance or substances that produce an inflammatory response characterised by erythema and induration, upon injection into the skin of normal guinea-pigs. These findings are in agreement with those of other workers who, using lymph node or peritoneal exudate lymphocytes from sensitised guinea-pigs were able to similarly demonstrate inflammatory activity in the supernatants from antigen-stimulated cultures of these cells (Bennett & Bloom, 1968; Dumonde et al., 1969; Heise & Weiser, 1969; Krejci et al., 1969; Pick et al., 1969). The inflammatory responses obtained in the present experiments reached a maximum at 4-6 hr and were characterised microscopically by an infiltration with polymorphonuclear and mononuclear cells occurring predominantly in the deeper portions of the dermis, mostly around blood vessels. These findings are essentially similar to those of Pick et al. (1969) and possibly of Dumonde et al. (1969) and Heise and Weiser (1969). Bennett and Bloom (1968) found only mononuclear cells present at 4 hr with neutrophils appearing later, as the reaction peaked at 8-12 hr. A mixture of polymorphonuclear and mononuclear cells was also noted by Krejci et al. (1969) but here the reaction was at its maximum at 24 hr.

The presence of a material inhibiting the migration of normal peritoneal exudate cells in the MIF Assay in those supernatants with inflammatory properties could indicate, as the results of Bennett and Bloom (1968) suggest, that MIF is the substance producing the inflammatory reaction *in vivo*. Probably, however, there is in these supernatants a large number of substances that have been synthesized by the stimulated lymphocytes and other factors besides MIF could be involved in the production of the red spot. If an actual substance SRF exists, distinct from MIF, lymphotoxin or chemotactic factor, such a separation must await more detailed knowledge of the physicochemical properties of these factors.

Cells in serum-free medium produce the factors, but do not show evidence of morphological transformation when examined at the end of the culture period. This should not be interpreted as indicating that the cells that transform are not those that produce the soluble factors. Production of the inflammatory substances assayed could occur early in the culture period, as Krejci et al. (1969) showed for SRF and Bennett and Bloom (1967) showed for MIF, and the cells subsequently degenerate before detectable changes occurred in their morphology. But equally, there could exist two populations of cells, one of small lymphocytes producing the soluble mediators and the other transforming and undergoing division when in suitable culture conditions. The relationship between the transformation that occurred in 20% serum and the presence of soluble factors in the supernatants might indicate that in those experiments where transformation did not occur, cells were incapable of survival or response to antigen, even in the presence of serum. Certainly, no dissociation occurred between the responses in serum and those in serum-free cultures, as might occur in the presence of serum inhibitory substances.

The number of animals fully investigated in this series is too small to draw any conclusions concerning the relative production of mediators in animals with different sensitivities to the antigen, i.e., to answer the question as to whether in animals strongly sensitive to tuberculin more cells respond with the production of mediators than in less sensitive animals. However, there does appear to be some correlation between cutaneous sensitivity, mitotic response and mediator production.

The main problem in this system as a model for the study of SRF in man concerns the in vitro assessment of transformation of stimulated lymphocytes. The method used here, the measurement of mitotic response, necessitates the presence of serum in the cultures initially, since in serum-free medium the cells do not divide. Thus, it is not a direct assessment of the response of cells producing the mediators actually assayed. In addition, as pointed out previously, cells may respond to antigen and begin to transform morphologically, producing soluble factors, but fail to reach the stage of cell division, perhaps because of changes occurring in the cultures. Furthermore, the assessment of transformation by the mitotic response as carried out here is not an absolute but rather a relative measure of responding cells. The final percentage figure is dependent upon the number of surviving lymphocytes and not just on the number of responding cells.

Thus, in the application of this technique to the investigation of SRF in man, it seems advisable to consider a more direct means of assessment of the degree of transformation of the sensitive lymphocytes in culture in response to antigen. One obvious possibility is the study of protein and ribonucleic acid (RNA) synthesis in the serum-free cultures by the use of radio-active isotopes.

B. EXPERIMENTS IN MAN

I. Early experiments on inflammatory factors produced by human peripheral blood lymphocytes stimulated with PPD.

These experiments were performed at approximately the same time as the initial experiments in guinea-pigs using lymphocytes from dextran-sedimented peripheral blood (Section A.I.) and before the development of the final protocol for the investigation of guinea-pig SRF.

Essentially, leukocytes obtained by the sedimentation of human peripheral blood from tuberculin sensitive donors were cultured with or without antigen, and supernatants from these cultures were assayed by:

(a) intradermal injection into the abdominal skin of normal guinea-pigs

(b) intraperitoneal injection into normal C57 black mice.

MATERIALS AND METHODS

Blood Donors: Peripheral venous blood was obtained from healthy adults of either sex exhibiting delayed hypersensitivity skin reactions to tuberculin.

Animals: Normal, unimmunized Hartley strain guinea-pigs weighing 500-700 g (A. & E. Farms, Altamount, New York, U.S.A.) and normal young adult C57 black mice (Jackson Laboratories, Bar Harbour, Maine, U.S.A.).

Skin Tests: Donors were skin tested with PPD (Connaught Laboratories) 1 TU by intradermal injection into the forearm. Reactions were assessed by measurement of the diameter of erythema and induration present at the injection site at 48 hr. Individuals not reacting to 1 TU were tested with increasing doses from 5 TU up to a maximum of 250 TU before being accepted as tuberculin-negative.

Preparation of Cells: Peripheral blood was obtained by venepuncture, the blood being drawn into a syringe containing sodium heparin (Connaught Laboratories) at a concentration of 10 units/ml of blood. A 6% solution of Dextran T110 (Pharmacia) in saline was added to the blood, 1 ml of dextran to 10 ml of blood, and the two were thoroughly mixed. The mixture was transferred to sterile plastic 15 x 125 mm culture tubes (Falcon Plastics) and the blood was allowed to sediment at 37° C for 1 hour. The leukocyte-rich plasma layer was then pipetted off, a drop was smeared upon a slide, fixed and stained for a differential count. A cell count was performed using a haemocytometer. The leukocytes obtained by this technique consisted of polymorphs 50 ± 12% S.D., lymphocytes 45.5 ± 14% and monocytes 4 ± 3%.

Culture Conditions: Peripheral blood leukocytes were cultured at concentrations from 0.6 - 25 x 10^6 lymphocytes/ml in RPMI 1640 (GIBCO) containing penicillin 100 units/ml and streptomycin 100 ug/ml (GIBCO) with 20% autologous plasma. Cultures were stimulated with PPD (Connaught Laboratories) 0.5 ug/ml and control tubes either had an equal volume of saline added to the cells in culture instead of antigen, or PPD was added after the cells had been killed by heat at 60° C for 30 min. Viability was checked by trypan blue exclusion. A further control consisted of culture medium with antigen added but without cells. Supernatants were removed after 3 days incubation at 37° C in sterile glass 100 x 13 mm culture tubes in triplicate in an humidified atmosphere of 5% CO_2 and air. The supernatants were cleared of cells by centrifugation at 1000 g for 10 min and were either stored at -20° C until used, or were dialysed at 4° C for 24 hr against 0.15 M sodium chloride and for 24 hr against distilled water. At the end of that time, a colloidal precipitate of protein had formed in the dialysis tube. The dialysed supernatants were divided into clear supernatant and colloid by centrifugation at 10,000 g for 20 min and were subsequently lyophilized separately and then stored at -20° C for future use. On two occasions after dialysis against distilled water alone, the dialysates were also collected, sterilised by Millipore filtration and then lyophilized and stored.

SRF Assay:

Guinea-pig experiments: Unconcentrated supernatants from stimulated and control leukocyte cultures were assayed by the intradermal injection of 0.1 ml of the fluid into the shaved abdominal skin of normal guinea-pigs. Lyophilized supernatants dissolved in 0.2 ml of RPMI 1640 and concentrated 5 to 40 times were also injected in volumes of 0.1 ml intradermally into the abdominal skin of normal guinea-pigs. Reactions were assessed for up to 24 hr and the diameter of induration and erythema or pallor produced was measured.

Mouse experiments: Unconcentrated supernatants from the leukocyte cultures were injected intraperitoneally in 1 ml aliquots into normal C57BL mice anaesthetized with ether. The animals were sacrificed at 8, 24 and 48 hr after the initial injection, each animal being injected intraperitoneally with 1 ml of warm RPMI 1640 1 hour prior to sacrifice. Subsequently, reactions were assessed at 24 hr only. After sacrifice, any fluid contained within the peritoneal cavity was withdrawn using a blood diluting pipette, a drop of the fluid was smeared upon a slide for later fixation and staining and the remaining fluid was used to carry out a white cell count using a haemocytometer.

Assessment of Transformation: This was undertaken in the form of a dose/response experiment using concentrations of PPD from 0.001 ug/ml to 10 ug/ml. Response of stimulated cultures was measured by the percentage of morphologically transformed lymphocytes in the cultures, 500 mononuclear cells being counted in methanol-fixed, Giemsa-stained smears from each culture.

RESULTS

The effect of increasing concentrations of PPD on the percentage of transformed cells in the cultures is depicted in Figure 26. Although the optimal dose of antigen on that basis is 10 ug/ml, there was considerable evidence for cell death in these cultures as compared to cultures with lower concentrations of PPD. Apart from blasts, small lymphocytes with pyknotic and irregular nuclei were seen as well as larger, poorly stained and apparently disintegrating cells with rounded or kidney-shaped nuclei and cytoplasm that failed to take up stain. In many of these, stain appeared to have leaked from the nucleus into the unstained cytoplasm. At lower concentrations of PPD, small lymphocytes more often appeared healthy and transformed, and dividing cells were visible. Since only healthy, recognisable mononuclear cells were counted in the assessment of the smears, the considerable degree of cell death at 10 ug PPD/ml would artificially raise the percentage of transformed cells.

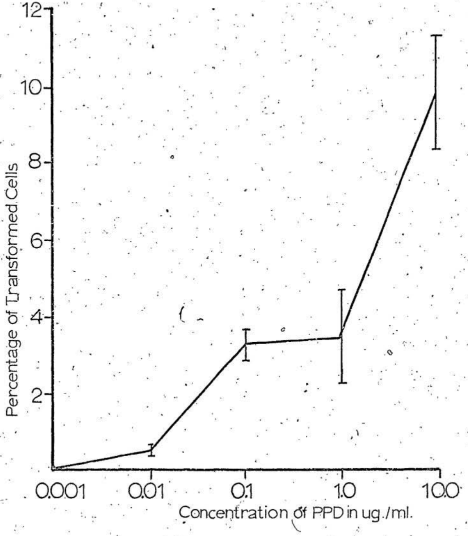


FIGURE 26. The effect of tuberculin concentration on the transformation of dextran-sedimented human peripheral blood lymphocytes from sensitised human donors. The range of results from triplicate cultures is shown.

TABLE 13

Inflammatory Activity in Supernatants from Sensitised Human Lymphocyte Cultures Stimulated by PPD and from Control Cultures

	SRF ASSAY		
	Mean Surface Area of Skin Reactions in sq mm ± S.D.		
No. of Expts.	Stimulated Cells	Unstimulated Cells	Killed Cells + Antigen
10	17.7 ± 8	10.2 ± 11	10.6 ± 20
Paired t test		p <0.2	p <0.4

Human SRF assayed in guinea-pigs.

As shown in Table 13, the injection of supernatants from sensitised human lymphocyte cultures intradermally into the shaved abdominal skin of normal guinea-pigs, failed to produce a significant inflammatory response. There was no significant difference between the effects of the supernatants from living cells incubated with PPD, from living cells incubated in the absence of PPD, or from killed cells incubated with antigen. In all cases, the reactions were small and pallor as often as erythema occurred with some slight induration.

The size of the reactions produced by the lyophilized supernatants are indicated in Table 14. In all categories, they were larger than those produced by unconcentrated supernatants. However, the major part of the inflammatory activity appeared to reside in the post-dialysis soluble fractions and not in the colloid nor in the dialysates. Although the largest reactions produced by this fraction were induced by supernatants from the stimulated cultures, considerable reactions were also found to occur with control supernatants. Thus, the difference between supernatants from cultures of viable cells incubated with and without antigen was significant only at the 5-10% level ($p < 0.1$). Reactions produced by supernatants from killed cells and the medium control incubated with antigen were significantly less than those elicited by supernatants from antigen-stimulated cultures ($p < 0.01$ and $p < 0.005$ respectively).

Reactions produced by the lyophilized colloidal material on intradermal injection were smaller than those produced by the soluble fractions and there was no significant difference between stimulated and control material.

The dialysates from the supernatants produced only a small reaction in the two experiments in which this was examined. Although this material had been concentrated 20 times, control supernatants failed to produce a reaction at all.

TABLE 14

Inflammatory Activity of Lyophilized Supernatants from Sensitised Human Lymphocyte Cultures Stimulated with PPD and from Control Cultures

Origin of Lyophilized Material		SRF ASSAY: Mean Surface Area of Skin Reactions in sq mm ± S.D.				
		No. of Expts.	Stimulated Cells	Unstimulated Cells	Killed Cells	Medium Control
Dialysed Supernatant	Fluid phase	11	129 ± 105	95 ± 64	49 ± 40	15 ± 27
				$p < 0.1$	$p < 0.01$	$p < 0.005$
	Colloid	9	39 ± 62	21 ± 27	26 ± 26	1 ± 2
				$p < 0.3$	$p < 0.5$	$p < 0.1$
Dialysates		2	16	–	–	–

Human SRF assayed in mice.

The intraperitoneal injection in mice of supernatants from the cultures indicated in Figure 27 demonstrated a peak in the inflammatory exudate produced, occurring at 24 hr, with supernatants from lymphocyte cultures stimulated with PPD. At other times and with control supernatants, the cellularity of the exudate remained approximately the same.

TABLE 15

Inflammatory Exudate Produced 24 hr after Intraperitoneal Injection in Normal Mice by Supernatants from Sensitised Human Lymphocyte Cultures and by Control Supernatants

Culture Supernatants		Stimulated	Unstimulated	Killed Cells	Medium Control
Cell Count x $10^3/mm^3$ ± S.D. for six animals		7.12 ± 2.3	2.3 ± 0.9	2.3 ± 0.6	3.4 ± 2
t test			p <0.01	p <0.01	p <0.05
Differential	Polymorphs	4	0	0	0
Count	Lymphocytes	74	88	91	95
%	Monocytes	22	12	9	5

When this exudate, occurring 24 hr after intraperitoneal injection of the culture supernatants, was examined in further experiments, it was found that supernatants from sensitive human lymphocyte cultures stimulated with PPD again produced exudates with significantly greater cellularity than did those from control supernatants (see Table 15). The differential counts carried out using a fixed and stained smear of the exudate did not appear to differ much between groups. There were more monocytes and fewer lymphocytes, and some polymorphonuclear leuko-

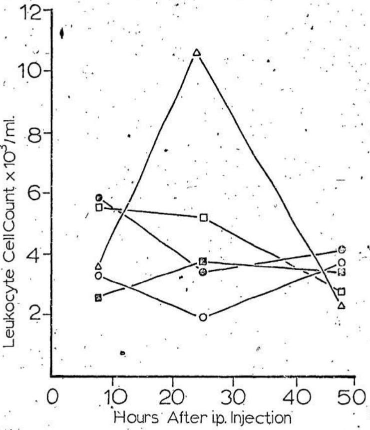


FIGURE 27. Inflammatory exudates produced in mouse peritoneal cavities at various times after the injection i.p. of supernatants from lymphocyte cultures.

Δ —— Δ cultures incubated with PPD 0.5 ug/ml

o —— o cultures incubated without antigen

o —— o killed cells incubated with PPD

□ —— □ culture medium control incubated with antigen

■ —— ■ culture medium control incubated without PPD cytes were present in the exudate developing in response to supernatants from stimulated cultures, as compared with the exudates produced using the control supernatants. However, the significance of these differences could not be determined because of the restricted number of satisfactory smears obtained for differential counting.

DISCUSSION

In the initial experiments into human SRF, carried out using unconcentrated supernatants from lymphocyte cultures stimulated with PPD or from the appropriate control cultures already described, significant inflammatory activity could not be demonstrated in the supernatants, on intradermal injection into the abdominal skin of normal guinea-pigs. There was no difference between the supernatants from either stimulated cultures or controls. There were three possible explanations for this failure. It could be that stimulation of the lymphocytes was not occurring and thus no SRF was being produced. A further possibility was that the amounts of SRF produced were insufficient to be demonstrated by the assay system used, being dispersed too rapidly following injection. Finally, species differences between human and guinea-pig SRF might prevent the former from being active in the assay animal. Such differences might require concentration of the SRF before its presence could be demonstrated, as appears to be the situation with human MIF assayed on guinea-pig macrophages (Rocklin et al., 1970).

In experiments where morphological evidence for transformation was sought in smears from the lymphocyte cultures, supernatants from apparently actively stimulated cultures still failed to produce an inflammatory response upon test injection.

The examination of concentrated supernatants from the cell cultures was complicated by the presence of serum in the culture medium to be dialysed and lyophilized. Attempts to overcome this complication by the testing of the three post-dialysis components of the supernatants, i.e., the colloidal precipitate formed during dialysis, the fluid phase left after the deposition of the colloid, and finally, the dialysate, appeared to indicate some extra inflammatory activity in the fluid phase of post-dialysis supernatants from stimulated cultures, when compared with those from control cultures. This difference was statistically significant and indicated a specific inflammatory component in these supernatants, when compared to the "non-specific" inflammation produced by injection of the lyophilized control supernatants. It did appear that the SRF remained in solution during dialysis and was not deposited in the colloid and did not pass across the dialysis membrane.

However, the clear demonstration of a specific SRF produced in response to antigen was obscured by the inflammation produced by control supernatants. This appeared to be the result of the presence of cells in the cultures rather than being due simply to the 20% plasma that was a component of the culture medium, since the medium and plasma control did not induce a significant inflammatory response. In this respect, non-specific inflammation could have been due to the considerable numbers of polymorphonuclear leukocytes present in the original cell suspension, since polymorphs have been shown to contain inflammatory substances (Ranadive & Cochrane, 1969). Another possibility was that the assay animal was reacting to the foreign substances being injected into it, mounting an inflammatory response against them.

In the experiments where an inflammatory peritoneal exudate had been raised by the injection of unconcentrated supernatants from lymphocyte cultures intraperitoneally into normal mice, there appeared to be evidence for the presence of inflammatory factors in the supernatants from stimulated cultures as indicated by the greater cellularity of the exudates induced. But once again the demonstration was complicated by the presence of "non-specific" inflammation induced by the control supernatants. In this case, exudates produced by the control supernatants from cell-free cultures exhibited considerable cellularity and here it was probably the presence of culture fluid in the peritoneal cavity that washed out mainly mononuclear cells. One would have expected to find more polymorphonuclear leukocytes if the control supernatants had excited an acute inflammatory response.

Certainly, in these experiments there appeared to be some evidence for the presence of inflammatory activity in supernatants from PPD-stimulated lymphocyte cultures. However, a clear and significant demonstration of antigen-specificity was not made because of the inflammatory reactions induced by supernatants from control lymphocyte cultures incubated in the absence of antigen.

II. Experiments carried out after the development of the guinea-pig system for the production and assay of SRF using peripheral blood lymphocytes.

These experiments were undertaken using a modification of the final protocol developed in the guinea-pig experiments (Section V). In that system, peripheral blood lymphocytes were obtained by centrifugation of the blood on ficoll/Hypaque, when the mononuclear cells remained at the interface between diluted blood and separating fluid. The red cells and polymorphonuclear leukocytes were found together deposited at the bottom of the tube in the ficoll/Hypaque. However, in the original description of the method of Böyum (1967) it was found that the polymorphs could be recovered from the red cell mass by a further sedimentation under gravity with dextran. It was considered, in the present work, that these polymorphs might be used in an *in vitro* assay of mediator production similar to the Leukocyte Migration Test (Søborg & Bendixen, 1967; Søborg, 1967). In that test, itself similar in technique to the MIF assay (David et al., 1964), peripheral blood leukocytes obtained by the sedimentation under gravity of peripheral blood, were, after washing, set up in capillary tubes and the migration of the leukocytes from sensitive and insensitive human donors was assessed in the presence and absence of specific antigen. In the original description, the hypersensitivity tested was to Brucellin and the antigen used was killed *Brucella Abortus* (Bang). Subsequently, it was shown that this system responded also to soluble antigens such as PPD (Clausen & Søborg, 1969; Rosenberg & David, 1970; Federlin et al., 1971; Mitchell et al., 1972) although there were initially difficulties with that demonstration (Kaltreider et al., 1969; Lockshin, 1969).

The leukocytes used in this test consist of a mixture of approximately equal parts of mononuclear and polymorphonuclear leukocytes, the predominant mononuclear cell being the lymphocyte. In the MIF assay it was shown that the indicator cell in that system, the macrophage, responded to the MIF produced by the antigen-stimulated sensitised lymphocyte with an inhibition of movement (Bloom & Bennett, 1966; Bennett & Bloom, 1967). Similarly, in the Leukocyte Migration Test (LMT) it might be assumed that the responding sensitive lymphocytes were producing a soluble factor inhibiting the indicator cells that were presumably the polymorphs. However, a clear demonstration of this phenomenon has not been carried out. Søborg (1969) and Clausen (1970) had shown that a mixture of polymorphs and lymphocytes was required for inhibition to occur in this test and that either cell type alone was not inhibited by antigen. In contrast, Rosenberg and David (1970) in experiments in which they analysed the migrating cell populations found that mononuclear cells and not polymorphs were inhibited. In addition, it had not been clearly demonstrated that the inhibition was caused by some soluble product of the antigen-stimulated lymphocyte. Rosenberg and David (1970) mentioned that in four preliminary experiments lymphocytes cultured with specific antigen (SK-SD) for 24-72 hr produced supernatants that inhibited the migration of leukocytes from other individuals compared with supernatants from lymphocytes incubated in the absence of antigen to which antigen was added later.

It thus appeared that, in the cell separation technique being utilised for the investigation of human SRF, there was a means by which certain questions related to the LMT might be answered. Specifically, it might be determined which cell type was acting as the indicator in this system and whether or not this inhibition was the result of a soluble factor produced by the stimulated sensitive lymphocyte. If these questions could be answered, the modified LMT could then be used as a convenient in vitro monitor of the activity of the stimulated lymphocytes in culture, in a manner analogous to the MIF assay used in Section V.

Therefore, in practical terms, the experiments to be described in the following two sections were, in fact, integrated by virtue of the cell separation technique employed, in that mononuclear cells were used for the in vitro production of SRF and polymorphs were used for the investigation and modification of the LMT. However, the experiments will be described separately as:

II (i) The development of the modified Leukocyte Migration Test — the Polymorphonuclear Leukocyte Migration Test (PLMT)

II (ii) The production and assay of human SRF with parallel assessment of in vitro mediator production by the PLMT.

II (i) The development of the modified Leukocyte Migration Test.

The method of Böyum (1967) for the separation of polymorphs and mononuclear cells from peripheral blood has been used in an investigation of the LMT by Clausen (1970). He demonstrated that populations of polymorphs and of lymphocytes from Brucellin-positive donors separated by Böyum's technique were not inhibited by the presence of the specific antigen, killed brucella bacteria, whereas mixtures of the two were. In the present experiments, the effect of the soluble antigen PPD upon mixtures of such pre-separated cells was initially examined, the technique being standardised by the addition of a constant proportion of mononuclear cells to the polymorphs. This test is referred to as the Direct LMT.

The effect of a metabolic inhibitor, puromycin, upon the antigen-specific inhibition of leukocytes in the LMT was investigated by Mitchell et al. (1972). The PPD-induced inhibition appeared to be blocked by the puromycin, although the interpretation of the results was made more difficult by the general inhibition of migration produced by the puromycin itself. These results suggest that some product of protein metabolism is responsible for the antigen-specific inhibitory response. A second metabolic inhibitor, actinomycin-D, which specifically binds to DNA thereby inhibiting DNA-dependent RNA synthesis was investigated here in its effect upon the LMT, since this was found not to inhibit normal migration to the same extent as puromycin.

Finally, mononuclear cells were obtained from tuberculin-positive and negative donors and were stimulated in culture with the antigen PPD. Cell-free supernatants from these and control cultures incubated without antigen were tested for their effects upon the migration of pure polymorphs in an Indirect LMT and on normal guinea-pig peritoneal exudate cells in an MIF assay.

MATERIALS AND METHODS

Donors: The blood samples were obtained from normal healthy human donors of either sex, aged from 20-50 years, without prior testing of their tuberculin sensitivity.

Skin Tests: These were carried out by the intradermal injection into the forearm of 0.1 ml of PPD (Connaught Laboratories). Initial testing was carried out using 1 TU and individuals not reacting were tested with 5 TU and subsequently retested up to a maximum of 250 TU before being considered tuberculin-negative. Reactions were assessed at 48 hr, measurements being taken of the diameter of erythema and induration present at that time.

Preparation of Cells: Thirty ml of peripheral venous blood was withdrawn into a disposable plastic syringe containing 1.5 ml of 2.7% EDTA in distilled water, pH 7.4. The blood was mixed thoroughly and then transferred to a sterile 250 ml plastic culture flask (Falcon Plastics) to which an equal volume of sterile isotonic saline was added. After mixing, the diluted blood was carefully layered using a Pasteur pipette onto the surface of 10 ml of separating fluid contained in each of two 50 ml glass round bottom centrifuge tubes. The separating fluid consisted of 10 parts Hypaque, 37.5% (Winthrop Laboratories) and 24 parts ficoll, 9% (Pharmacia). The tubes were carefully balanced and then centrifuged at 400 g at the interface for 24 min at room temperature. The diluted plasma was then removed down to the cell fraction at the interface and was retained. The mononuclear cell fraction from each tube was drawn off using a Pasteur pipette and was at once diluted with 30 ml of cold HBSS containing sodium heparin 10 units/ml, in a sterile plastic conical bottom centrifuge tube. The cells were then spun at 500 g for 15 min at 4° C. The red cell/polymorph fraction was resuspended in an equal volume of the diluted plasma (retained from the original separation) and with 6% dextran 110 (Pharmacia), 1 ml to 5 ml of the original fraction. This was left to sediment in 15 x 125 mm glass tubes at a 45° angle at 4° C.

The mononuclear cells, after centrifugation were resuspended in fresh HBSS with heparin and a cell count using a haemocytometer and an assessment of viability by trypan blue exclusion was carried out (lymphocyte viability was >99%). Two drops of the cell suspension were incubated with one drop of heat-inactivated FCS (BBL) and one drop of a suspension of latex particles in saline (Bacto-Latex, Difco, 1:25 dilution) for 30 min at 37° C. After resuspending, the cells were examined in a slide chamber using a Wild M40 inverted microscope. Cells were assessed with respect to size, shape and intracellular inclusions, especially the latex particles. Lymphocytes which did not take up latex particles made up 92.64 ± 4.05% of the total whilst mononuclear phagocytic cells contributed 7.78 ± 4.35%. A little of the original mononuclear cell suspension was also smeared on a serum-coated slide, dried, fixed with methanol and later stained with Giemsa. The cells were stored at 4° C until used, never for more than 1 hr.

After 1-2 hr of sedimentation of the red cell/polymorph fraction, the leukocyte-rich supernatant was removed and the cells were washed twice in cold HBSS containing heparin 10 units/ml, at 250 g for 5 min at 4° C. After pipetting off the cell-free supernatants, the polymorphs, which were usually contaminated with red cells, were resuspended in 1 ml of distilled water by gentle pipetting for 15 sec and then 10 ml of Eagles MEM (GIBCO) containing 20% heat-inactivated FCS was added, the capped tube was inverted gently several times and the cells were spun down at 250 g for 5 min at 4° C. Finally, the leukocytes were resuspended in 5 ml of MEM containing 20% FCS. A cell count and assessment of viability by trypan blue exclusion was carried out. This was invariably greater than 90%. A differential count under phase after incubation with latex demonstrated that polymorphonuclear leukocytes comprised 98.75 ± 1% of the cells with lymphocytes making up the remainder, 1.25 ± 1%. The cells were stored at 4° C until used.

Culture Conditions: Mononuclear cells were cultured at a cell concentration of 2.5 x 10^6 cells per ml in 2 ml of RPMI 1640 (GIBCO) containing 20% heat-inactivated FCS (BBL) and with penicillin 100 units/ml and streptomycin 100 ug/ml (GIBCO). Cultures were stimulated with excipient-free PPD (Parke-Davis & Co., Montreal, Canada) in Hepes-buffered 199 (GIBCO), 0.025 - 2.5 ug/ml in a dose/response experiment and with 2.5 ug/ml and 10 ug/ml in experiments to produce supernatants for assay in the LMT. Control cultures received an equal volume (0.1 ml) of the Medium 199. Cells were incubated in duplicate cultures (in triplicate in the case of the dose/response experiment) in 75 x 13 mm sterile plastic culture tubes (Falcon Plastics) at 37° C in 5% CO_2 and air in an humidified atmosphere. For the dose/response cultures, cells were incubated for 120 hr. On all other occasions, at 24 hr 1 ml of the supernatant was removed from each culture and duplicate samples were pooled and stored at -20° C. Fresh warm RPMI 1640 with the appropriate amount of antigen and 20% serum was added back to the tubes which were returned to the incubator. At 72 hr, a further 1 ml of supernatant was removed from each culture, duplicates pooled and stored at -20° C.

Assessment of Stimulation: Uridine-6-^{3}H (New England Nuclear-Canada Ltd., Montreal, Quebec, 25.3 c/mM) 1 uC/ml of culture was added to each tube in a volume of 0.05 ml. For the dose/response experiment, thymidine-2-^{14}C (New England Nuclear, 60 mC/mM) 0.05 uC/ml of culture was added in a volume of 0.1 ml. Cells were exposed to the isotope for 2 hr at 37° C and at the end of the labelling period, cultures were harvested immediately by a technique similar to that of Dutton and Page (1964). The cells were transferred to glass 13 x 100 mm culture tubes by two washes with 4 ml of ice-cold isotonic saline and after washing the cells once more in 5 ml of cold saline at 500 g for 5 min at 4° C, the nuclear protein was precipitated by 3 ml of cold 5% trichloracetic acid (TCA). The precipitate was washed twice in 5 ml of cold 95% methanol and dried at 37° C. The precipitate was solubilized in 0.5 ml of NCS Tissue Solubilizer (Amersham/Searle Corporation, Toronto, Canada) for 18 hr at 37° C. The contents of each tube was transferred to scintillation counting vials by two washes with 6 ml aliquots of Spectrofluor PPO (Amersham/Searle) scintillation fluid. The vials were counted in a Nuclear Chicago Mark I Liquid Scintillation Counter. The results were corrected for background activity and quenching using an external standard and expressed in disintegrations/min.

The Leukocyte Migration Test: In the Direct LMT, after initially determining the optimal percentage, 10% of the pre-separated mononuclear cells were added to polymorphs previously stored at 4° C and the two were mixed by gentle pipetting in a 13 x 75 mm plastic culture tube (Falcon Plastics). In the Indirect LMT polymorphs alone were used. The cell suspensions were spun down at 250 g for 5 min and the supernatant completely removed. The cells were then carefully resuspended in Hepes-buffered Eagles MEM (GIBCO) containing 20% heat-inactivated FCS at a concentration of 30 x 10^6 cells/ml and were kept cold in ice. Capillary tubes (Non-heparinised Microhaematocrit, I.D. 1.1-1.2 mm, Fisherbrand) were filled with the cell suspension and sealed at one end with vinyl plastic putty (Critoseal, Sherwood Medical Industries Inc., St. Louis, Missouri) taking care to resuspend the cells with a Pasteur pipette after filling each pair of tubes. The capillary tubes were stored upright in ice in a small plastic tube. After filling and sealing all the tubes required, they were spun at 130 g for 2 min in a clinical centrifuge. The tubes were cut at the interface and the portions containing the cells were placed in Mackaness-type chambers (Disposable 'Lexy' Culture Chambers, Mini-Lab, Duvernay, Laval, Quebec, Canada), two capillaries in each chamber, held in place with silicone vacuum grease (Dow Corning Corporation). A sterile coverslip was sealed onto the top of each chamber with paraffin wax (Histowax). For the direct test, the chambers were filled with a 1:1 mixture of Hepes-buffered MEM and 199 (GIBCO) with penicillin 100 units/ml and streptomycin 100 ug/ml, 20% heat-inactivated FCS and excipient-free PPD (Parke-Davis), 10 ug/ml or 100 ug/ml (Mitchell et al., 1972). Some initial experiments were also carried out using PPD (Connaught) but its use was discontinued when it was shown that a considerable proportion of the inhibition produced by this preparation was the result of preservatives present in it. Control tubes were filled with medium and serum alone. Where the effects of a metabolic inhibitor on the antigen-specific inhibition was assessed, the medium contained, in addition, 1 uM actinomycin-D (Sigma Chemical Company,

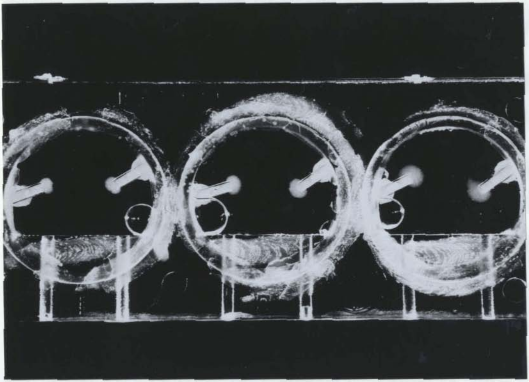


FIGURE 28. The leukocyte migration test showing inhibition of migration of the polymorph/mononuclear cell mixtures in the presence of PPD 100 ug/ml (the chambers contain from left to right, 100 ug PPD/ml, 10 ug PPD/ml and no antigen).

St. Louis, Missouri, U.S.A.). In the Indirect test, supernatants from stimulated and control lymphocyte cultures incubated with PPD for 24 hr and 72 hr were added to the chambers. The chambers were then sealed with paraffin wax and incubated at 37° C for a total of 18 hr (Figure 28). The area of migration at 6 hr and 18 hr or at 18 hr only was projected using a Zeiss Projecting Microscope onto a sheet of stiff paper, 0.4 mm thick, and this was outlined. The area of migration was subsequently cut out and weighed. The results were expressed as:

$$\% \text{ Migration} = \frac{X}{Y} \times 100$$

where X = the weight of the area of migration in the presence of antigen or in supernatants from antigen-stimulated cultures

Y = the weight of the area of migration in the absence of antigen or in supernatants from control (unstimulated) cultures.

MIF Assay: The peritoneal exudate cells for this assay were prepared using the technique described in Section V, using a 2.5% hydrolysed starch solution to induce the exudate. The chambers were set up as indicated previously and supernatants from the stimulated and control lymphocyte cultures incubated for 72 hr with antigen were tested. The area of migration was assessed using the Zeiss Projecting Microscope and the results were expressed as a percentage migration compared to control.

RESULTS

The Direct Leukocyte Migration Test.

In an experiment (Figure 29) undertaken to demonstrate the percentage of mononuclear cells required to be added to the polymorphs to obtain inhibition of migration in the test in response to PPD, it was

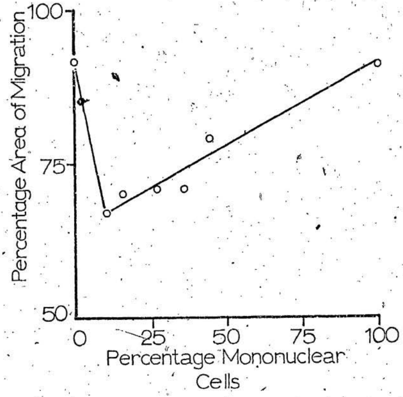


FIGURE 29. The area of migration in the presence of 100 ug PPD/ml is plotted as a percentage of control (in the absence of antigen) for reconstituted mixtures of pre-separated polymorphs and mononuclear cells in various proportions from a tuberculin-sensitive donor. Results are the mean of duplicate assays.

FIGURE 30

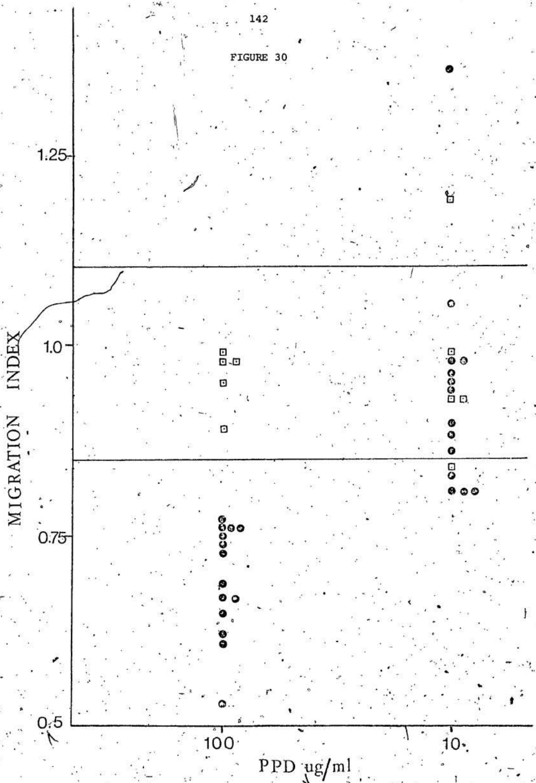
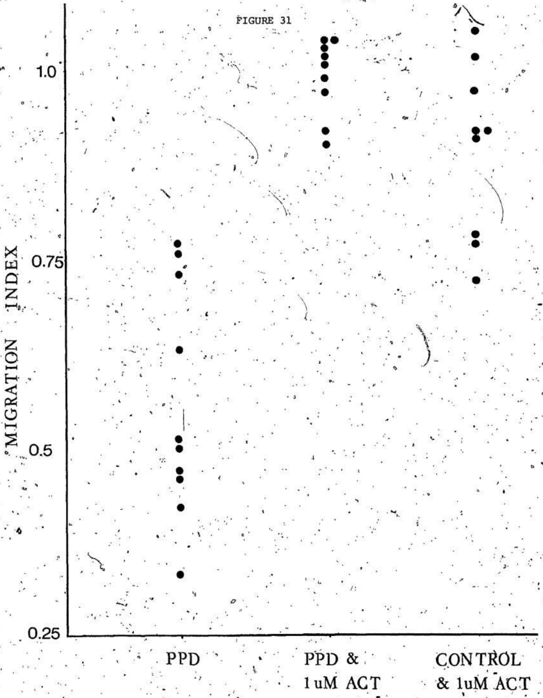


FIGURE 31



found that 10% of mononuclear cells produced the greatest inhibition. This proportion of mononuclear cells was used subsequently.

The migration of the 10% mononuclear/polymorph cell mixtures prepared from donors of unknown cutaneous tuberculin reactivity were then examined in the direct LMT in the presence of PPD 10 ug/ml and 100 ug/ml and in its absence. The results were as indicated in Figure 30 and are here expressed with regard to the subsequently determined cutaneous sensitivity of the donors. When the cells from tuberculin-positive individuals were exposed to 100 ug PPD/ml, the mean migration as a percentage of control was 68 ± 7% (S.D.) whereas for tuberculin-negative donors the migration was 98 ± 4%. These results are significantly different ($p < 0.001$). At the lower antigen dosage, 10 ug PPD/ml, there was no difference between the migration of cells from tuberculin-positive and negative donors (94 ± 14% and 98 ± 13% respectively). Subsequently in the test an area of migration in the presence of 100 ug PPD/ml less than 84% of control was considered to indicate significant inhibition (this being less than t = -3 standard deviations from the mean for tuberculin-negative donors).

When the effects of 1 uM actinomycin D upon the PPD-specific inhibition of the mononuclear/polymorph mixtures was examined (Figure 31) it was found that actinomycin D blocked the inhibition whilst reducing control migration only slightly. Thus, in the presence of 100 ug PPD/ml, the mean area of migration was 56 ± 15% and this was increased by the addition of 1 uM actinomycin D to 106 ± 22%. These results are significantly different ($p < 0.001$). The area of migration of the control cells was reduced slightly by the presence of actinomycin D, to 90 ± 12% ($p < 0.1$). Thus, the antigen-specific inhibition of migration was prevented by the blocking of m-RNA synthesis. That RNA synthesis was halted by this concentration of actinomycin D was shown in experiments where the incorporation of uridine-6-^{3}H in leukocyte cultures was reduced from 15×10^{3} dpm/10^{6} cells/hr to 19 dpm/10^{6} cells/hr.

The Indirect Leukocyte Migration Test.

In this series of experiments, supernatants from antigen-stimulated and control lymphocyte cultures were tested for their effects upon the migration of pure polymorphs prepared from peripheral blood. Polymorphs were obtained from donors irrespective of their tuberculin sensitivity after it was demonstrated that these cells from tuberculin-positive donors were not inhibited in the direct LMT by the presence of 100 ug PPD/ml (Table 16).

TABLE 16

The Migration of Pure Polymorphs and Mixtures of 10% Mononuclear Cells and Polymorphs in the Direct LMT in the Presence of PPD 100 ug/ml

Migration of Polymorphs	Migration of Mononuclear/ Polymorph Mixtures	t Statistic
92.6 ± 6%	69.4 ± 5.4%	$p < 0.005$

A dose/response experiment was carried out assessing the incorporation of thymidine-2-^{14}C into mononuclear cells at doses of excipient-free PPD up to 2.5 ug/ml (Figure 32). A straight line relationship was found to occur. Subsequently, in the preparation of supernatants for assay in the LMT, 2.5 ug PPD/ml and 10 ug PPD/ml were used, the latter because this was one of the doses used in the direct LMT.

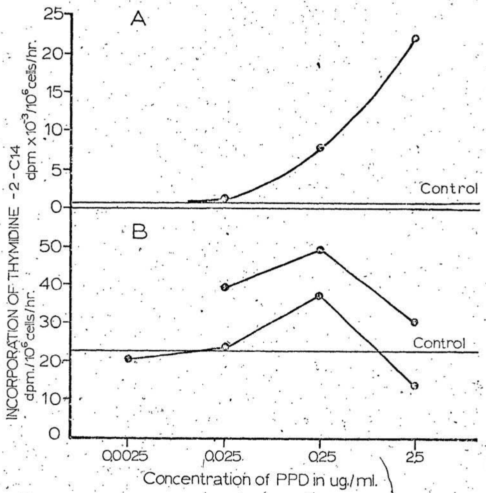


FIGURE 32 (a) & (b). The effect of PPD concentration on the incorporation of thymidine-2-^{14}C into mononuclear cells from tuberculin-sensitive donors at 5 days in serum-containing (a) and serum-free (b) cultures. Results are the mean of triplicate cultures. Two experiments are shown in (b).

The cell-free supernatants prepared from lymphocyte cultures after 24 hr and 72 hr incubation with or without PPD were tested for their effects upon the migration of polymorphs in the indirect LMT and against normal guinea-pig peritoneal exudate cells in the MIF assay. These results, with data obtained from the study of uridine-6-^{3}H incorporation in the mononuclear cell cultures, are presented in Table 17. The experiments were carried out before skin testing the donors with PPD but the results are presented with respect to the subsequently determined cutaneous tuberculin sensitivity. In the direct LMT there was significant inhibition of cells from tuberculin-positive donors in the presence of 100 ug PPD/ml whereas inhibition did not occur with cells from tuberculin-negative individuals ($p < 0.001$). At the lower antigen dose of 10 ug PPD/ml, inhibition was not less than 84% of control and was not considered significant, although there was a difference in the migration of positive and negative donor cells ($p < 0.1$). Uridine-6-^{3}H incorporation in stimulated cultures was slightly increased over control levels with cells from positive donors when compared with cultures of tuberculin-negative cells, i.e., there was some increase in RNA synthesis in those cultures. But the difference between the two groups was only significant at the 10% level.

In the assay for a supernatant factor inhibiting the migration of polymorphs in the indirect LMT, it was found that inhibition occurred with supernatants from 24 hr cultures of cells from tuberculin-positive individuals stimulated with 2.5 ug PPD/ml. When the test was read at 6 hr the difference between positive and negative groups was highly significant ($p < 0.001$). By 18 hr however, the significance of the difference between positive and negative donors was less ($p < 0.02$).

TABLE 17

Indirect LMT and MIF assays carried out on supernatants from 24 hr and 72 hr mononuclear cell cultures from tuberculin-positive and tuberculin-negative donors incubated with 2.5 ug PPD/ml (A) and 10 ug PPD/ml (B). Assays read at 6 hr and 18 hr or at 18 hr only. Results expressed as % area of migration as compared to migration in control (antigen-free) supernatants. Direct LMT performed with 10% mononuclear/polymorph mixtures of donor cells. RNA synthesis in the mononuclear cell cultures assayed as incorporation of uridine-6-^{3}H in dpm/5.10^6 cells/hr in stimulated (A & B) compared to control (C) cultures. Monocytes made up 3.9 ± 1.4% of the mononuclear cells in cultures from tuberculin-positive donors and 5.8 ± 2.4% of these from tuberculin-negative donors. These differences were not significant. Results are expressed as the mean ± S.D. for 12 samples in the tuberculin-positive group and for 8 samples in the tuberculin-negative group.

TABLE 17. See opposite page for legend.

ASSAY				TUBERCULIN +ve	TUBERCULIN -ve	t test
SUBJECT NOS.				6	4	
DIRECT L.M.T.	M.I. PPD 100ug/ml			0.67 ± 0.06	0.92 ± 0.1	p= 0.001
DIRECT L.M.T.	M.I. PPD 10 ug/ml			0.89 ± 0.06	1.01 ± 0.15	p= 0.1
^{3}H-URIDINE INCORPN	A PPD 2.5 ug/ml			1.45 ± 0.68	0.84 ± 0.23	p= 0.1
^{3}H-URIDINE INCORPN	B PPD 10 ug/ml			1.54 ± 0.97	0.72 ± 0.19	p= 0.1
INDIRECT L.M.T.	24 hr supernatants	6 hr reading	A	70 ± 11	99 ± 7	p= 0.001
INDIRECT L.M.T.	24 hr supernatants	6 hr reading	B	66 ± 15	95 ± 11	p= 0.02
INDIRECT L.M.T.	24 hr supernatants	18 hr reading	A	76 ± 10	98 ± 11	p= 0.02
INDIRECT L.M.T.	24 hr supernatants	18 hr reading	B	84 ± 15	100 ± 10	p= 0.1
INDIRECT L.M.T.	72 hr supernts.	18 hr reading	A	88 ± 31	126 ± 71	p= 0.3
INDIRECT L.M.T.	72 hr supernts.	18 hr reading	B	71 ± 19	134 ± 81	p= 0.1
MIF ASSAY	72 hr Supernts	18 hr reading	A	94 ± 11	98 ± 23	P<0.8
MIF ASSAY	72 hr Supernts	18 hr reading	B	101 ± 17	98 ± 14	P<0.9

When the stimulus to the lymphocytes in culture was PPD 10 ug/ml, the difference between the two groups was still significant when the test was read at 6 hr but by 18 hr, once more the difference was less marked ($p < 0.1$). Supernatants from 72 hr cultures demonstrated little difference between the tuberculin-positive and negative donors, whether assayed in the LMT or as MIF, except in the case of supernatants from lymphocyte cultures stimulated with 10 ug PPD/ml and assayed by the indirect LMT. These results are given as the migration at 18 hr since inhibition was not greater at the earlier time. In addition, the aim of this portion of the experiment was a comparison of the results of the LMT with the MIF assay, which is usually read at 18-24 hr. Experiments using 24 hr culture supernatants where inhibition of migration occurred in the indirect LMT similarly failed to produce inhibition in the MIF assay.

When the period of incubation in the LMT was extended for periods longer than 18 hr it could not be demonstrated conclusively that the inhibition reversed itself and that migration of the polymorphs occurred once more. The edges of the migrating cells became more diffuse and were difficult to assess.

Cell viability at 18 hr by trypan blue exclusion was greater than 90% and there was no difference in the viabilities of inhibited and control cells.

DISCUSSION

The Leukocyte Migration Test was originally introduced by Søborg and Bendixen (1967) as an <u>in vitro</u> assessment of delayed hypersensitivity to brucellin using as antigen a suspension of the killed <u>Brucella abortus</u>

PPD ug/ml

bacteria. It was initially uncertain whether hypersensitivity to soluble antigens such as PPD could be assessed by this technique. Thus, Kaltreider et al. (1969) and Lockshin (1969) were unable to demonstrate the specific inhibition of sensitised cells using soluble PPD that Clausen and Søborg (1969) reported. Subsequently, other groups (Rosenberg & David, 1970; Federlin et al., 1971; Mitchell et al., 1972) demonstrated significant inhibition of movement of sensitised leukocytes in response to PPD using concentrations from 100–300 ug PPD/ml.

In the present series of experiments, it has also been possible to demonstrate antigen-specific inhibition of migration of peripheral blood leukocytes from tuberculin-positive donors in the presence of 100 ug PPD/ml (Figure 30). In addition, this inhibition was demonstrated using mixtures of pre-separated and reconstituted mononuclear and polymorphonuclear leukocytes in the ratio of 10% mononuclear cells to 90% polymorphs. The mononuclear cells added consisted of 92.6 ± 4% small lymphocytes. These results, using pre-separated cells and a soluble antigen are in agreement with the results of Clausen (1970) who used a similar cell separation technique and a particulate antigen, killed Brucella bacteria to demonstrate the inhibition of cells from brucellin-positive individuals.

Mitchell et al. (1972) used puromycin to block the antigen-specific inhibition produced by PPD in the LMT, demonstrating that this inhibition was the result of an active process requiring protein synthesis. Using different concentrations of puromycin they were able to show that the abolition of the antigen-specific inhibition was dose-dependent. However, they did not include data in their paper to demonstrate that protein synthesis had been blocked by the levels of inhibitor that they used. In addition, the migration of control cells, incubated with puromycin but in the absence of PPD was significantly inhibited, sometimes by as much as 50%. The sensitivity of the assay and the possibility of showing significant antigen-specific inhibition in this situation may be considerably reduced.

The prevention of PPD-specific inhibition in the LMT by actinomycin D in the experiments described above (Figure 31) occurred in the absence of a significant reduction of migration of control cells in the presence of the inhibitor. The dose of actinomycin D used here reduced RNA synthesis, as assessed by uridine-6-^{3}H incorporation, to insignificant levels. It might be surmised that m-RNA required for protein synthesis in normal cell movement is still present within the migrating cells but new species of m-RNA possibly required for the synthesis of factors inhibiting polymorph movement are not produced in the presence of the actinomycin.

In fact, the means by which inhibition of migration in the LMT is produced has not been agreed upon in the past. Søborg (1969) presented evidence that when sensitive and non-sensitive peripheral blood leukocytes were cultured in the same chamber with the antigen, killed Brucella bacteria, the migration of both cell types was inhibited, without direct contact between sensitive and non-sensitive cells. In addition, the migration of sensitive lymphocytes, not inhibited when cultured alone with specific antigen, was inhibited when the lymphocytes were cultured in the same chamber as either sensitive or non-sensitive leukocytes. Clausen (1970), using peripheral blood leukocytes separated by the method of Böyum (1967) demonstrated that isolated polymorphs and isolated mononuclear leukocytes from brucellin-positive persons did not show antigen-induced inhibition of migration. A mixture of 85-90% sensitised lymphocytes and 10-15% polymorphs did not show antigen-induced inhibition either. However, a mixture of equal proportions of pre-separated polymorphs and mononuclear cells or lymphocytes showed antigen-induced inhibition of migration to the same degree as non-separated leukocytes. In contrast to this, Rosenberg and David (1970) in an analysis of the migrating cells in the LMT suggested that it was the mononuclear cells and not the polymorphs that were inhibited by antigen.

On the basis of the MIF assay, where it has been demonstrated that sensitised lymphocytes respond to the presence of antigen with the production of a substance or substances, MIF, that inhibit the movement of normal unsensitised macrophages (David et al., 1964; David, 1966; Bloom & Bennett, 1966), it might be assumed that a similar phenomenon occurs in the LMT. Thus, one would expect that sensitised lymphocytes would respond to the presence of antigen with the production of a factor inhibiting the movement of either themselves, monocytes or polymorphs. Rosenberg and David (1970) mentioned in their work on the LMT that in four preliminary experiments lymphocytes cultured with specific antigen (SK-SD) for 24-72 hr produced supernatants that inhibited the migration of leukocytes from other individuals compared with supernatants from lymphocytes incubated in the absence of antigen to which antigen was added later.

In the present experiments, it was shown that the migration of polymorphs prepared by ficoll/Hypaque separation of peripheral blood was not affected by the presence of PPD at concentrations where mixtures of mononuclear cells and polymorphs were significantly inhibited in their migration. In addition, cell-free supernatants from cultures of sensitive and insensitive mononuclear cells consisting of >90% lymphocytes, incubated in the presence of the specific antigen, PPD, produced significant inhibition of migration of pure polymorphs only when the supernatants were obtained from cultures of cells from tuberculin-positive donors (Table 17). These results suggest that, as occurs in the MIF assay, sensitised lymphocytes respond to the presence of antigen with the production of a factor inhibiting the movement of polymorphs.

But an important question remains unanswered as to the nature of the factor involved in this reaction. Is it the same as MIF? Attempts to show a correlation with MIF activity in this system were unsuccessful, but possibly for reasons unrelated to the nature of the factor itself. Thus, at 72 hr the supernatants were only marginally active in the LMT (Table 17) and since Rocklin et al. (1970) have shown that human MIF requires concentrating 4-5 times in order for it to be assayed in a guinea-pig system, it is perhaps not surprising that no MIF activity could be demonstrated here. The correlation of the MIF assay with the LMT will be considered further in the final section of this thesis.

A more serious question concerns the possibility that the factor influencing polymorph movement in the LMT is not of that group of substances, the putative soluble mediators of delayed hypersensitivity to which MIF belongs. Thus, antibody may bind to and thus affect the migration of polymorphs (Ishizaka et al., 1970) and may be produced in the LMT in response to antigen (Wasserman & Pakalén, 1965). Søborg (1971) found no correlation between the presence of agglutinating antibodies toward <u>Brucella</u> bacteria and inhibition in the leukocyte migration test in man following primary immunization with the bacteria. In experiments carried out in our laboratory (Warrington & Sheikh, 1973, unpublished observations) examination of polymorph suspensions from the LMT experiments, comparing PPD-inhibited and control cells, for the presence of IgG on the cell membranes by immunofluorescence using fluorescein-labelled goat anti-human IgG were uniformly negative. However, further clarification regarding the nature of the inhibitory factor is necessary.

The presence of most of the inhibitory activity in supernatants from 24 hr cultures, as compared to 72 hr cultures, would suggest that the inhibitory factor is produced early in the response of lymphocytes to antigen. The fact that inhibition is most clearly demonstrated at 6 hr as opposed to 18 hr suggests that the inhibition is at least partially reversible. An alternative suggestion that might explain both phenomena is that the inhibitory material is labile and is broken down in culture at 37° C. However, the inhibitory activity appeared to be stable on storage at 4° C for several days and at -20° C for some weeks. In addition, the inhibitory activity present in serum-containing supernatants was not affected by dialysis against 0.15 M saline for 24 hr and distilled water for 24 hr with subsequent lyophilization. Such reconstituted supernatants produced marked inhibition of migration in the indirect LMT.

The occurrence of an early infiltration with polymorphs of variable degree in the tuberculin reaction (Gell & Hinde, 1951; Wesslén, 1952; Boughton & Spector, 1963) and the presence of a significant proportion of polymorphs in the cutaneous reaction induced by SRF (Bennett & Bloom, 1968; Dumonde et al., 1969; Krejci et al., 1969; Pick et al., 1969) when considered in the light of the above demonstration of a soluble lymphocyte product affecting the migration of polymorphs _in vitro_, suggests that this cutaneous infiltration might similarly be due to such a factor, produced by sensitised lymphocytes reacting with antigen in the skin. This might be considered to be affecting polymorphs in an analogous way to that in which MIF modifies the migration of mononuclear phagocytic cells, as was discussed previously in the introduction, immobilizing cells in the area in which the specific immune response is occurring.

II (ii) The production and assay of human SRF with parallel assessment of in vitro mediator production by a Polymorphonuclear Leukocyte Migration Test.

In this final series of experiments the production of human SRF was carried out using a modified version of the system developed in the investigations of guinea-pig SRF described in Section A.V, i.e., where mononuclear cells from ficoll/Hypaque-separated peripheral blood were cultured in serum-free medium and the cell-free supernatants, after dialysis, were concentrated by lyophilization and assayed by intradermal injection into normal guinea-pigs. The modifications introduced consisted of:

(a) the parallel in vitro assay of mediator production in the supernatants was carried out using the modified indirect LMT already described in Section B.II (i). This was used instead of the MIF assay that was used in the guinea-pig experiments. This test will subsequently be referred to as the Polymorphonuclear Leukocyte Migration Test (PLMT).

(b) transformation of the lymphocytes in serum-free cultures was assessed directly by the measurement at 72 hr of the incorporation of radio-active precursors thymidine-^{14}C and uridine-^{3}H into the cells in the cultures from which supernatants to be tested were derived, instead of indirectly by the mitotic response in parallel serum-containing cultures. The latter method of assessment suffered from the obvious disadvantages of being carried out under different culture conditions, i.e., in the presence of serum that might modify the response (see Section IV (ii)) and at a later time when deterioration of cell viability might have occurred.

MATERIALS AND METHODS

Donors: Blood was obtained from normal healthy adults of either sex, aged 20-50 years, without prior testing of their cutaneous reactivity to tuberculin.

Animals: These were male Hartley guinea-pigs (A & E Farms) weighing 500-700 g for assaying SRF.

Skin Tests: Donors were skin tested after blood had been donated for the experiments. The procedure was as outlined in the preceding Section B.II (i).

Preparation of Cells: This was carried out using the technique already described in Section B.II (i), separating peripheral blood leukocytes into mononuclear and polymorphonuclear cell fractions by the method modified from Böyum (1967).

Culture Conditions: After spinning down the mononuclear cells at 250 g for 5 min, the cells were resuspended in 2 ml of either RPMI 1640 (GIBCO) containing penicillin 100 units/ml and streptomycin 100 ug/ml (GIBCO) or in this culture medium supplemented with 20% heat-inactivated human cord serum (prepared in the laboratory of Dr. W.H. Marshall), at a cell concentration of 1 x 10^6 cells/ml for dose/response experiments or at 2.5 x 10^6 cells/ml in cultures for SRF production. The antigen used, PPD (Parke-Davis) was added to the cultures to give a final concentration of 0.025 - 2.5 ug/ml in the dose/response experiments and 0.25 and 2.5 ug/ml for the SRF experiments. Control cultures received an equal volume of the culture medium in which the PPD was dissolved. SRF production was carried out in serum-free medium. Cells were incubated, in sterile glass 100 x 13 mm culture tubes at 37° C in 5% CO_2 and air in a humidified atmosphere for 3 days for SRF production and for 5 days for dose/response experiments. Cultures were incubated in duplicate in the former experiments and in triplicate in the latter.

Preparation of SRF: One ml aliquots of the supernatants from stimulated and control cultures were withdrawn daily using a sterile disposable 1 ml pipette and were stored at 4^{o} C. An equal volume of fresh warm (37^{o} C) medium containing the appropriate amount of antigen was added back to the cultures. On the third day, cultures were centrifuged at 200 g for 5 min at room temperature, the culture medium was drawn off, avoiding disturbing the cells and the cell button was resuspended in fresh warm RPMI 1640, ready for the addition of isotope for the assessment of stimulation. Supernatants, pooled from duplicate cultures over the 3 day incubation period, after centrifugation at 16,000 g for 20 min, were pipetted off, PPD was added to give equal concentrations in all tubes and they were dialysed against 0.15 M saline at 4^{o} C for 24 hr and against distilled water for 24 hr. After sterilising by filtration using an 0.22 u cellulose filter (Millipore Ltd.) the supernatants were lyophilized and stored at -20^{o} C until used.

Assessment of Stimulation: After resuspending the cells at the end of the culture period in 2 ml of warm RPMI 1640, the duplicate cultures were divided, 1 ml to each of 4 tubes and 1 uC of uridine-6-^{3}H (New England Nuclear, 25.3 c/mM) or 0.05 uC of thymidine-2-^{14}C (New England Nuclear, 60 uC/mM) were added to each 1 ml culture in a volume of 0.05 ml. Cells were exposed to the isotope for 2 hr and harvested using the technique described in Section B.II (i), modified from that of Dutton and Page (1964). Results, corrected for background and for quenching by the use of an external standard, are expressed as dpm/10^{6} cells/hr unless stated otherwise.

SRF Assay: The lyophilized supernatants were dissolved carefully in 0.2 ml of RPMI 1640 (Hepes buffered, GIBCO) containing antibiotics. These supernatants (concentrated 40 times) were drawn carefully into 1 ml tuberculin syringes through a 20 G needle, subsequently exchanged for a 30 G ½" disposable needle. A volume of 0.05 ml of each supernatant tested was injected intradermally into the shaved abdominal skin of normal guinea-pigs. Reactions were observed for up to 24 hr but were measured at their peak, at 4-6 hr, by the diameter of the erythema and induration and the increase in double skin thickness, measured using the Schnelltäster (System Kröplin). The remaining concentrated supernatant was diluted up to a total volume of 0.7 ml (concentration now 5 times the original) in RPMI 1640 supplemented with 20% FCS for use in the PLMT.

Histology: Biopsies were taken from skin reactions at 6 hr, fixed in formaldehyde fixative and stained with haematoxylin and eosin.

Polymorphonuclear Leukocyte Migration Test: This was carried out using the technique described in Section B.II (i), using pure polymorphs and assessing the migration of these cells in the concentrated supernatants remaining after the SRF assay.

Direct Leukocyte Migration Test: This was carried out using the technique described in Section B.II (i), with the modification that in some assays, 20% serum was not added.

RESULTS

The lymphocyte response to PPD in vitro in serum-free and serum-containing cultures as assessed by the incorporation of labelled precursors.

The incorporation of thymidine-2-^{14}C into lymphocytes cultured for

5 days in the presence and absence of 20% heat-inactivated human cord serum was assessed in the form of dose/response experiments, stimulating the cells with excipient-free PPD at concentrations from 0.0025 - 2.5 ug/ml. It can be seen (Figure 32 a & b) that in the presence of serum, incorporation of isotope increases linearly to a maximum at 2.5 ug PPD/ml. However, in the absence of serum, incorporation of isotope is reduced by a factor of about a thousand. At this level, counts are barely above background and the difference in incorporation at the various concentrations of PPD are probably not significant. Even so, there does appear to be a peak in two different experiments at 0.25 ug PPD/ml, i.e., at one-tenth the optimal concentration of antigen in serum-containing cultures.

In similar experiments using the culture system utilised for the production of SRF, i.e., with a cell concentration of 2.5×10^6 cells/ml stimulated for 3 days with 0.25 or 2.5 ug PPD/ml, a similar discrepancy between the incorporation of thymidine-2-^{14}C in serum-free and serum-containing cultures was seen.

The results of the two experiments presented in Table 18 indicate that DNA synthesis was much reduced in the serum-free cultures. When RNA synthesis was assessed by the incorporation of uridine-6-^{3}H into the cells, it was found that in the presence of serum, both stimulated groups A & B showed an increase over control levels, the highest incorporation occurring with 2.5 ug PPD/ml. However, in serum-free medium, uridine incorporation in stimulated cultures was in one experiment just marginally increased over control levels and in the other was less than control. In addition, the greatest incorporation of isotope occurred with 0.25 ug PPD/ml.

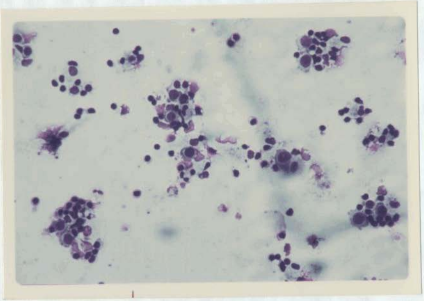


FIGURE 33. Transformed lymphocytes (lymphoblasts) present in serum-free cultures stimulated with PPD. Giemsa. x 320.

These results might be considered to be due to poor survival of the cells in the absence of serum. But in contrast to the viability of guinea-pig lymphocytes in serum-free medium (Section V) a considerable proportion of human lymphocytes were still viable, as assessed by trypan-blue exlusion, at the end of the 3 day incubation period (50 ± 26%). There was no evidence for an increase in cell death at the higher antigen concentration (2.5 ug PPD/ml) and although the viability of the cells in culture varied from experiment to experiment, it was relatively consistent within a particular experiment.

Smears prepared from some of the serum-free cultures, fixed with methanol and stained in Giemsa, showed that transformed lymphocytes were present in the antigen-stimulated cultures and occasionally some of these blasts were in the process of dividing (Figure 33).

TABLE 18

Incorporation of Thymidine-2-^{14}C or Uridine-6-^{3}H into Peripheral Blood Mononuclear Cells Incubated for 3 days in Serum-Free Medium or Medium Supplemented with 20% Human Cord Serum, Stimulated with 0.25 ug PPD/ml (A), 2.5 ug PPD/ml (B) or in the Absence of Antigen (C). Results from Two Experiments (i) and (ii).

		Isotope Incorporation in dpm/10^6 cells/hour					
		Serum-Free Cultures			Serum-Containing Cultures		
		A	B	C	A	B	C
Thymidine-2	(i)	17	21	12	223	551	22
-^{14}C Incorpn.	(ii)	2	7	2	115	230	4
Uridine-6	(i)	2.06	1.49	1.44	11.6	26.3	4.2
-^{3}H Incorpn. x 10^3	(ii)	7.1	4.6	9.9	10.4	18.1	6.6

The production and assay of human SRF with a parallel in vitro assessment of transformation by DNA and RNA synthesis and of mediator production by the PLMT.

A total of 18 individuals were tested of whom 10 were male. The tuberculin sensitivity of the donors, as assessed by the cutaneous tuberculin reaction, is indicated in Table 19, a positive reaction being considered to be one with >5 x 5 mm erythema and induration.

There was no significant difference between the percentage of phagocytic mononuclear cells in the initial cell cultures in groups (a) and (b). The numbers are too small for the significance of the difference to be determined in groups (c) and (d).

The incorporation of isotope, either as thymidine-2-^{14}C or uridine-6-^{3}H into the cells stimulated with 0.25 ug PPD/ml (A) or 2.5 ug PPD/ml (B) and in control cultures without antigen (C) is also given in the table. The levels of DNA synthesis in these cultures, as assessed by thymidine-2-^{14}C incorporation, are too low for any significance to be attached to differences between the groups. There is very little DNA synthesis occurring in these cultures. RNA synthesis, as measured by uridine-6-^{3}H incorporation is greatest in groups (a) and (b) in cultures stimulated with a low dose of PPD, 0.25 ug/ml. At the higher concentration of antigen a depression in the mean uridine incorporation has occurred. This depression was not correlated with any differences in the cell viability of the cultures, as assessed by trypan blue exclusion. This difference between cultures A and B in the two groups was just significant ($p < 0.1$). However, uridine incorporation in stimulated cultures as compared to that in control cultures was not significantly different. The results in groups (c) and (d) are few in

TABLE 19

Cutaneous Tuberculin Sensitivity and the Transformation in Response to Antigen of Mononuclear Cells from Tuberculin-Positive and Tuberculin-Negative Donors. Cells were stimulated with 0.25 ug PPD/ml (A) or 2.5 ug PPD/ml (B) for 3 days in Serum-Free Medium. Results are expressed as the mean ± S.D.

No. of Subjects	Tuberculin Sensitivity	% Monocytes	Transformation: Thymidine Incorporation dpm/10^6 cells/hr			Uridine Incorporation dpm/10^6 cells/hr x 10^3		
			A	B	C	A	B	C
(a) 6	1 TU	6.8 ± 3	22 ± 20	19 ± 15	9 ± 10	3.1 ± 1.6	2.6 ± 1.1	2.9 ± 1.1
(b) 8	5 TU	8.8 ± 5	11 ± 5	15 ± 6	7 ± 4	3.3 ± 1.1	2.8 ± 1.4	3.0 ± 1.6
(c) 2	100 TU	10.3	9	4	7	4.7	3.6	4.8
(d) 2	Negative	3.6	4	8	5	3.8	3.1	5.1

FIGURE 34. The inflammatory response produced by the injection of supernatants from stimulated human lymphocyte cultures from a tuberculin-positive donor (upper right and middle sections). There is a lack of inflammation both with the unstimulated (control) supernatant (upper left section) and in the lower sections where the supernatants injected were derived from cultures of cells from a tuberculin-negative donor. Reactions were photographed at 6 hrs.

number but here the incorporation of the labelled precursors was reduced in all cultures to which antigen was added as compared to control, especially with the higher dose of PPD and to a greater extent in cultures of cells from tuberculin-negative individuals.

When the cell-free concentrated supernatants from these cultures were tested for their ability to induce an inflammatory reaction in the abdominal skin of normal unimmunized guinea-pigs, it was found that, in the tuberculin-negative group (d), the supernatants failed to produce an inflammatory response, once the initial reaction at the injection site had disappeared. Thus, by 5-6 hr the injected areas showed no reaction at all.

In contrast to this, a marked inflammatory response was induced by supernatants from cultures of cells from tuberculin-positive donors (Figure 34). These results are indicated in Table 20.

TABLE 20

The Inflammatory Reaction in sq mm of Erythema and Induration Induced by Supernatants from Mononuclear Cell Cultures from Tuberculin-positive and Tuberculin-negative Donors Incubated for 3 Days with PPD 0.25 ug/ml (A), 2.5 ug/ml (B) or in the Absence of Antigen (C). Results are expressed as the mean ± S.D. for 16 samples in each group.

Supernatant	Inflammatory Reaction in sq mm: Tuberculin + ve	Tuberculin - ve
A	86 ± 53	0
B	58 ± 64	0
C	45 ± 62	0

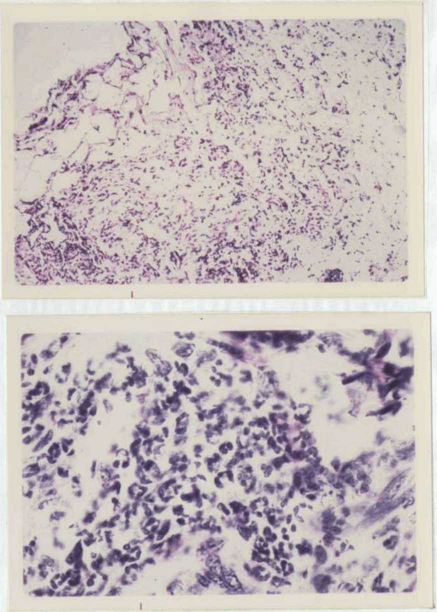


FIGURE 34 a & b: The infiltration of cells in the inflammatory response induced by supernatants from stimulated cultures of cells from a tuberculin-positive donor. x 32
x 320

There was a greater inflammatory activity in the supernatants from stimulated cultures than there was in supernatants from control cultures and this difference was most marked with supernatants stimulated with 0.25 ug PPD/ml, where it was statistically significant ($p < 0.005$) using a paired t test. For B supernatants the difference was not significant. In fact, the control supernatants on several occasions produced quite marked inflammatory reactions.

The inflammatory response induced by the active supernatants was characterised histologically by the presence of a rather marked infiltration of the skin by leukocytes, predominantly polymorphs but with about 25% mononuclear cells (Figure 34 a & b). The infiltration was present throughout the dermis and was often perivascular in distribution. It was not predominantly located in the deeper regions of the dermis. In control reactions there was a scanty infiltration with some polymorphs and mononuclear cells.

The results from the PLMT assays were analysed in two groups, for A and B supernatants. These were subdivided into SRF +ve and SRF -ve depending upon whether or not the supernatants in question had induced an inflammatory response in the SRF assay greater than that induced by the control supernatant. The results of this analysis are presented in Table 21.

It was found that there was no evidence for inhibition of migration in the PLMT being correlated with the presence of inflammatory activity in the supernatants. In fact, there appeared to be some slight enhancement of migration in the presence of SRF +ve supernatants although the differences were barely significant ($p < 0.1$ for A supernatants and $p < 0.05$ for B supernatants). It might be that the polymorph migration inhibitory factor, in contrast to MIF, requires the presence of serum for its formation and this appeared to be the case in an examination of the direct LMT undertaken in serum-free medium and in 20% foetal calf serum. The leukocytes in the test (10% mononuclear cells and 90% polymorphs) from tuberculin positive donors were exposed to 100 ug PPD/ml for 18 hr. The migration in 20% serum was inhibited (mean % migration from two experiments was 62%) whereas in serum-free medium there was no inhibition (mean 101%) although migration overall was reduced non-specifically by the lack of serum, i.e., migration of control cells was only about 50% of that of the control in 20% serum. However, as can be seen from Figure 35, in the assay of the supernatants in the SRF series of experiments, there was significant inhibition of migration in the PLMT induced by some of the supernatants tested, whereas others induced a marked stimulation of migration.

TABLE 21

The % Migration in the PLMT in the Presence of Supernatants from Stimulated and Control Cultures of Cells from Tuberculin-positive and Tuberculin-negative Donors, Subdivided on the Basis of the Inflammatory Activity of the Supernatants. Results are expressed as the mean ± S.D. for 22 samples in the SRF +ve group and for 12 samples in the SRF -ve group.

	% Migration in the PLMT		
Supernatants	SRF +ve	SRF -ve	t test
A	118 ± 20	96 ± 22	$p < 0.1$
B	112 ± 23	90 ± 17	$p < 0.05$

On the basis of the results in the PLMT (Figure 35), it can be seen that the experimental supernatants can be subdivided into three

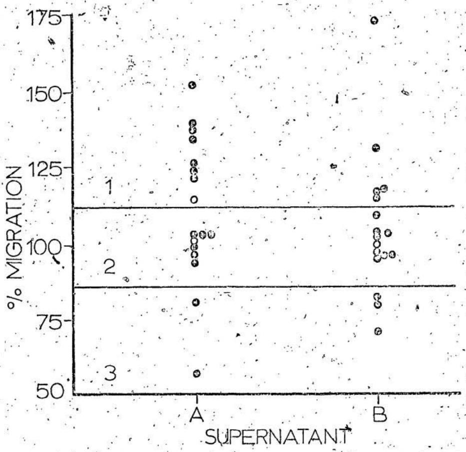


FIGURE 35. The % migration of polymorphs in the PMT in the presence of supernatants from cultures of cells from tuberculin-positive donors (closed circles) and tuberculin-negative donors (open circles) stimulated with PPD 0.25 ug/ml (A) or 2.5 ug/ml (B). The lines indicate t = ± 2 standard deviations from the mean for tuberculin-negative donors in the indirect LMT (Section B.II (ii)).

groups, as indicated in the above Figure, depending upon whether migration of the cells in the stimulated culture supernatants A or B was significantly different from that in control supernatants.

When the inflammatory activity of the supernatants in the SRF assay from stimulated and control cultures were then compared in each of the three groups, the results were as given in Table 22.

TABLE 22

A Comparison of the Inflammatory Activity of Supernatants from Stimulated and Control Cultures of Lymphocytes from Tuberculin-positive Donors, Subdivided on the Basis of Their Effects upon the Migration of Polymorphs in the Leukocyte Migration Test. The number of samples in each group is seen in Figure 35. Results are expressed as mean ± S.D.

	SRF ASSAY		
	Diameter of Erythema and Induration in sq mm		
Supernatants	PLMT Groups: (1)	(2)	(3)
Stimulated	81 ± 63	63 ± 50	69 ± 92
Control	17 ± 21	22 ± 23	159 ± 50
t test	p <0.005	p <0.01	p <0.2

There appears to be a correlation between enhancement of migration in the PLMT and antigen-specific inflammation in the SRF assay. The difference between the inflammatory activity of stimulated culture supernatants and that of unstimulated control culture supernatants is highly significant (p <0.005) in the group demonstrating stimulation of migration in the PLMT. The difference was still significant, but at a lower level (p <0.01) with supernatants from group (2), where migration in the stimulated culture supernatants was not significantly different from that in control supernatants. However, where significant inhibition occurred in the PLMT (group 3) there was no significant difference between the inflammatory activity in stimulated and control culture supernatants. This was because the inflammation produced by the control supernatants was considerably increased in this group.

If one then compares the inflammatory activity of supernatants A, B & C from the tuberculin-positive donors in groups (1) & (2) who were sensitive to either 1 TU or 5 TU on cutaneous testing, it is found that A supernatants produced a mean reaction of 67 ± 41 sq mm erythema and induration, B supernatants gave a reaction 69 ± 75 sq mm and C supernatants produced a reaction of 12 ± 15 sq mm. These differences are statistically significant for the stimulated culture supernatants (A & B) compared to control (C) (for A $p < 0.001$ and for B $p < 0.05$).

DISCUSSION

The initial dose/response experiments (Figure 32) and the experiments in which transformation, as assessed by DNA and RNA synthesis, were compared in serum-free and serum-containing cultures (Table 18) indicate that the lack of serum in cultures profoundly affects the response of cells to antigen. Thus, there is an overall depression of RNA and DNA synthesis in the serum-free cultures and an antigen-specific stimulation of nucleotide synthesis is not apparent. Indeed, at the higher antigen dose, 2.5 ug PPD/ml, there is evidence for a depression in isotope incorporation, supported by the shift of the dose/response curve in serum-free medium to the left. This would appear to suggest that a toxic or inhibitory effect of the antigen occurs in the absence of serum. Of course, the interpretation of the uridine-incorporation data is complicated by the fact that an unknown proportion of this incorporation is being carried out by phagocytic mononuclear cells in the cultures. Thus, it cannot be determined whether the toxic or inhibitory effect of the antigen is exerted upon the lymphocytes or monocytes or both in these cultures.

A similar phenomenon was noted in the incorporation data in the experiments for SRF production (Table 18). Here there was no significant difference between isotope incorporation in stimulated and control cultures in the two groups (a) and (b) where sufficient data was available for comparison. However, there was a depression in RNA synthesis in the cultures exposed to the higher dose of PPD (2.5 ug/ml) as compared to that in the cultures stimulated with a low dose of antigen. There was no evidence that the depression in uridine incorporation in these cultures was the result of a decrease in cell viability caused by the toxic effects of the antigen. Viabilities as assessed by trypan-blue exclusion were not significantly different in either antigen stimulated cultures or control cultures.

Despite the general depression of metabolism as assessed by the incorporation of isotope in the serum-free cultures, there was still evidence for the production of soluble factors by the cells in response to antigen. Thus, cells from tuberculin-positive donors upon exposure to PPD released inflammatory factors into the culture medium. This was in contrast to cells from tuberculin-negative donors, where there was no evidence for a release of SRF. The inflammatory factor was not dialysable and upon injection into the skin of normal guinea-pigs produced a reaction characterised by erythema and induration. This reached a peak at about 4-6 hr and then faded. It was characterised histologically by a marked infiltration with polymorphonuclear leukocytes and a smaller proportion of mononuclear cells. In this respect the reaction differed somewhat from that induced by guinea-pig SRF, where mononuclear cells formed about 50% of the cellular infiltrate (Figure 24). In addition, the infiltration was found throughout the dermis, although still more profound in the deeper regions and was mainly perivascular.

The antigen-specificity of the release of the SRF was more apparent (i.e., differed more significantly from control) when cells were stimulated with the lower antigen dose, 0.25 ug/ml (Table 20). This would suggest that the inhibitory effects of the higher dose of antigen, 2.5 ug/ml are reflected in the depression of incorporation of uridine-6-^{3}H, and in a reduction of mediator production.

Non-specific inflammatory activity has been demonstrated to be produced by glass adherent phagocytic cells in culture (Heise & Weiser, 1969; Maillard et al., 1972) and vaso-active factors are released upon cell death in lymphocyte cultures (Maillard et al., 1972). But in the current experiments, no correlation was found between the original % of phagocytic mononuclear cells in the cultures and the presence of inflammation in the SRF assay. Similarly, cell viability was not decreased in those cultures from which active supernatants were obtained. In addition, the data from the studies of uridine incorporation suggest that SRF is a product of active metabolism and is not released secondary to cell death. Thus, in cultures where RNA synthesis is reduced, there is less evidence for SRF production.

There was no evidence for a significant difference in uridine-6-^{3}H incorporation or SRF production in the two tuberculin-sensitive groups

(a) & (b), i.e., those reacting positively to 1 TU and 5 TU respectively on cutaneous testing. In part this may be a result of the use of serum-free media in the mononuclear cell cultures, where serum factors or other cell types possibly modifying the in vivo immune response are not present. The numbers in the remaining groups (c) & (d) were too small for any statistical analysis although it should be noted that in the tuberculin-negative groups, uridine incorporation in cultures containing antigen was quite markedly depressed and SRF was not produced.

The antigen-specific release of SRF appeared to correlate with the lack of inhibition of migration of polymorphs in the PLMT and was most apparent in supernatants that actually enhanced migration (Table 22). This might suggest that, in the absence of serum, a factor is produced by sensitive lymphocytes in response to antigen that stimulates the migration of polymorphs. However, the significance of this is difficult to assess. But it was apparent that supernatants stimulating migration were more often produced by lymphocyte cultures exposed to the optimal dose of antigen, 0.25 ug PPD/ml (Figure 35) and this might suggest that the stimulation of migration is a result of the antigen-specific response of the lymphocyte. The rather profuse infiltration with polymorphs in the inflammatory reactions induced by human SRF could be explained by an enhancement of polymorph movement, although this evidence can only be indirect until it is shown that guinea-pig polymorphs also respond in a similar manner to this factor derived from human lymphocyte cultures. In addition, such stimulation of movement could presumably only occur before the changes in vascular permeability induced by other factors, e.g., SRF either directly or possibly by activation of the kinin system (Maillard et al., 1972), caused an influx of plasma factors allowing inhibition to replace stimulation.

But it is clear that serum is required for the production of the polymorph inhibitory factor and is not just necessary for the demonstration of its presence, as the results comparing the direct LMT in serum-free and serum-containing medium might suggest, since 20% foetal calf serum was added to the serum-free supernatants assayed in the PLMT. Thus, it is possible that the necessary serum-factors were present in the original mononuclear cell cultures from which the inhibitory supernatants were obtained, perhaps through some error in the washing procedure. If so, this might explain the presence of inflammatory activity in the control (unstimulated culture) supernatants in this group, since it was shown in Section B.I that the dialysed and lyophilized soluble phase of serum-containing supernatants from unstimulated cultures possessed considerable inflammatory activity (Table 14). Apart from this possible explanation, there were no other obvious differences between cultures producing inhibitory or stimulating supernatants. The % monocytes in the original cell cultures, the viability of those cultures and the uridine incorporation data did not differ significantly. Obviously the role of serum factors in the LMT needs to be more clearly defined, since the requirement for serum indicates an important difference between this test and the MIF assay, where inhibitory supernatants can be produced by stimulated lymphocytes in serum-free cultures (Rocklin et al., 1973).

However, it does appear clear that, in the group of cultures of peripheral blood mononuclear cells from tuberculin-positive donors where there is no evidence for the production of polymorph migration inhibitory factors, there is a significant release of inflammatory activity in response to the specific antigen. The factor or factors producing the inflammation are not dialysable, may be lyophilized and stored at -20° C and produce an inflammatory response with erythema and induration, on intradermal injection in normal unimmunised guinea-pigs. The reaction reaches a peak at 4-6 hr and is characterised at the cellular level by an infiltration, predominantly perivascular, of polymorphs and mononuclear cells. In this respect, the human SRF appears to be similar to the guinea-pig SRF described previously, in cultures of peripheral blood lymphocytes (Section A.V) and lymph node and peritoneal exudate lymphocytes (Pick et al., 1969).

GENERAL SUMMARY AND CONCLUSIONS

In the initial investigations in guinea-pigs it was shown that peripheral blood mononuclear cells from tuberculin-positive animals stimulated in vitro with PPD, released into the medium a factor or factors that induced an inflammatory response upon intradermal injection into normal unimmunised guinea-pigs. The characteristics of this inflammatory substance appeared to be similar to the skin reactive factor (SRF) described by Pick et al. (1969) in cell-free supernatants from stimulated cultures of guinea-pig lymph node and peritoneal exudate lymphocytes. It was not dialysable and induced a response characterised by erythema and induration reaching a peak at 4-6 hr, with a cellular infiltrate consisting of polymorphs and mononuclear cells. The presence of this factor in the supernatants was found to correlate in vitro with the presence of inhibition of macrophage migration in the MIF assay. The SRF was produced in serum-free cultures in the absence of blast-transformation and subsequent division of the stimulated lymphocytes. However, it cannot be concluded from this that the cells producing these factors are not those that would proceed to transformation under appropriate culture conditions. There appeared to be some correlation between the ability of the lymphocytes to transform and divide in the presence of serum and the release of SRF in serum-free cultures of the same cells. The process of transformation here may be an indicator of the ability of the lymphocytes to form and release the soluble factors.

The importance of culture conditions in determining if transformation occurs was demonstrated by the occurrence of factors inhibiting that process in sera of certain guinea-pigs. That these might be important in the comparison of the in vitro and in vivo immune responses was indicated by the results of experiments comparing the growth and division of lymphocytes in sera from different animals. The appearance of these factors might be linked to the process of ageing in the animal and this is a field in which there is scope for further investigation. In addition, the toxic or inhibitory effects of specific antigen upon the transformation of sensitive lymphocytes was demonstrated and this effect appeared to be more prominent in the absence of serum. Whether lymphocytes or phagocytic mononuclear cells were mainly affected by the inhibitory effects of serum or antigen could not be decided in the current experiments. However, the interaction of the two cell types in the immune response to antigen is so intimate that a clear distinction may not be possible. But here also further study would be fruitful.

The marked effects of the lack of serum on the immune response of sensitised lymphocytes to PPD was further demonstrated in experiments in man, where transformation, as assessed by DNA and RNA synthesis was significantly depressed in the absence of serum. In addition, there was suggestive evidence that a toxic or inhibitory effect, even in quite low concentrations, of PPD occurred in serum-free cultures.

Of course, a lack of serum may affect immune responses to antigen quantitatively so that, for example, an antigen-specific release of soluble factors can still be demonstrated, in the serum-free cultures, by concentrating the factors involved. But in the investigations of the leukocyte migration test reported here, it appeared that a lack of serum might possibly alter an immune response qualitatively as well. Thus, in the presence of serum, it was possible to demonstrate that sensitised mononuclear cells were able, in the presence of antigen, to inhibit the migration of polymorphs and that this process required measurable RNA synthesis for its occurrence. In addition, in cell-free serum-containing supernatants of sensitised lymphocyte cultures stimulated with PPD, it was possible to demonstrate a factor that was probably responsible for the inhibition of migration. This factor was not found in culture supernatants of cells from tuberculin-negative donors. However, in the absence of serum, inhibition of migration was not demonstrated, either when mixtures of sensitised mononuclear cells were mixed with polymorphs in the presence of antigen, or when cell-free supernatants from serum-free cultures of antigen-stimulated sensitive lymphocytes were examined. In fact, in a number of instances, these serum-free supernatants appeared to cause actual enhancement of polymorph migration. But until the significance of this apparent stimulation of migration is determined, the possibility still exists that the difference between the results in serum-free and serum-containing media is quantitative rather than qualitative. Even if this is so, the LMT still exhibits a marked difference from the MIF assay in this respect, where serum is not necessary for the production of the mediator. This is especially important because of the marked increase in the use of the leukocyte migration test in recent years as an *in vitro* correlate of the cell-mediated immune response. Evidently it is necessary for a further clarification of the nature of the inhibitory factor in this test.

Finally, the existence of an inflammatory factor in the cell-free supernatants of stimulated peripheral blood mononuclear cells from tuberculin-positive human donors was clearly demonstrated. This factor is not dialysable and induces an inflammatory reaction characterised by erythema and induration upon injection into the skin of normal unimmunised guinea-pigs. The cellular infiltration consists of polymorphs and mononuclear cells which are mainly perivascular in distribution and the reaction itself reaches a peak at from 4-6 hr. In most respects, the human inflammatory factor appears similar to the guinea-pig SRF already described. As was indicated in the introduction to this thesis, whether or not there is a discrete SRF molecule must await further characterisation of its physico-chemical properties. Recent estimates of the size of guinea-pig SRF suggest that it is between 35,000 - 42,000 (Maillard et al., 1972), i.e., approximately the same as MIF (Remold et al., 1971) and indeed they may be identical. Its function in the tuberculin reaction may be restricted to inducing changes in the small vessels, especially by increasing permeability and even this action may be indirect, through an activation of the kinin system or Hageman factor, as suggested recently by Maillard et al. (1972). Other lymphocyte products, distinct from SRF are likely to be involved in the induction of a cellular infiltrate of mononuclear cells and possibly polymorphs, e.g., chemotactic factor, MIF, factors affecting polymorph migration described here. In addition, materials contained within the infiltrating cells and other factors derived from the constituents of plasma are likely to be involved in the induction of the delayed hypersensitivity inflammatory response.

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