

Studies on the Transfer Factor of Delayed Hypersensitivity

EFFECT OF DIALYSABLE LEUCOCYTE EXTRACTS FROM PEOPLE OF KNOWN TUBERCULIN SENSITIVITY ON THE MIGRATION OF NORMAL GUINEA-PIG MACROPHAGES IN THE PRESENCE OF ANTIGEN

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Summary. The hypothesis that transfer factor (TF) is an antibody-like molecule, possibly related to the T-cell receptor, has been explored. Experiments to test the hypothesis have involved determining whether TF can act as an antigen-dependent macrophage inhibition factor (MIF).

Peritoneal exudate cells from normal guinea-pigs were migrated from capillary tubes in the presence and absence of PPD and of 'dialysable' TF prepared from the buffy-coat cells of people with strong positive or negative delayed reactivity to this antigen. At a low concentration of PPD (8 $\mu\text{g}/\text{ml}$) there was a small but consistent inhibition of migration in the presence of positive, but not negative, TF. At a higher concentration (20 $\mu\text{g}/\text{ml}$) the situation was complicated by enhancing and inhibitory effects of PPD on migration occurring in the absence of TF. In half the experiments where PPD had little effect strong inhibition was seen in the presence of positive TF. Where PPD produced enhancement there was no further effect of TF; where PPD caused inhibition TF consistently overcame this, positive TF being more potent.

These experiments cannot establish unequivocally an antibody-like structure for TF. Peritoneal exudates contain some lymphocytes and TF, whatever its nature, could be transferring sensitivity to these so that they produce MIF in the presence of antigen. Indeed, a correlation was observed between small effects of positive TF in the absence of PPD and the actions of PPD itself. There are difficulties in accepting that TF is a form of antigen, but this is an attractive solution from the standpoint of molecular weight.

INTRODUCTION

It has been known for 20 years that delayed hypersensitivity of a lasting nature can be transferred in man by injection of extracts of white cells from the blood of individuals who are positive skin reactors to the particular antigen (Lawrence, 1969a,b). The active principle in such preparations is known as transfer factor (TF). Most work on TF has

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involved injection of lysates prepared by freezing and thawing the cells and treating the resulting viscous mixture with DNase. The evidence for antigenic specificity appears strong. Transfer does not normally occur when a negative donor is used; degrees of donor and transferred sensitivities tend to go in parallel; where more than one skin sensitivity has been tested the pattern of sensitivity as a whole was transferred.

More recently it was reported that on dialysis of the cell lysate TF appears in the dialysate (Lawrence, Al-Askari, David, Franklin and Zweiman, 1963). Although the evidence relating to specificity is less extensive the phenomenon observed on injection of such dialysates appears to be the same as that seen with the whole extract (Lawrence, 1969a, b). The dialysate has been shown to confer, in an apparently specific manner, the ability to produce a transformation or macrophage inhibition factor (MIF) response when the recipient's lymphocytes are subsequently cultured with antigen (Fireman, Boesman, Haddad and Gitlin, 1967; Lawrence and Valentine, 1970; Levin, Spitler and Fudenberg, 1973).

Because of its specificity TF is presumably antigen, antibody or informational nucleic acid. For reasons which will be set out in the discussion an antibody-like structure is an attractive hypothesis, and this suggests a possible relationship between TF and antigen-dependent MIF. This type of MIF is thought to be produced by lymphocytes involved in delayed hypersensitivity, and to possess a combining site while being of smaller molecular size than conventional immunoglobulin; and it has the property of inhibiting the migration of macrophages in the presence of antigen (Amos and Lachmann, 1970; Lachmann, 1971). Experiments were therefore carried out to see whether 'dialysable' TF had the same inhibitory property. If it did, this could provide the basis of an *in vitro* assay for TF.

MATERIALS AND METHODS

Preparation of transfer factor

Donors of blood for preparation of TF were from the laboratory staff. A Heaf tuberculin skin-test was given and responses were graded from zero (negative) to four (strong positive). Donors were chosen from the extreme categories. The principal negative donor (DF), unlike some skin-test negative individuals, was completely unresponsive to PPD in transformation tests.

The method used for preparation of TF was similar to that described by Lawrence and Al-Askari (1971). Venous blood (45 ml) was taken into preservative-free heparin (15 units/ml). One-half the volume of 3 per cent gelatin (Davis Gelatin, Warwick) in phosphate-buffered saline was added and the blood left to sediment for 30 minutes at 37°. The supernatant was removed and, after sampling for a white-cell count, was centrifuged at 220 *g* for 10 minutes at room temperature. The pellet was resuspended in 1.3 ml of 0.9 per cent NaCl and transferred to a Pyrex glass tube. This was placed in a freezing mixture of 90 per cent ethanol and dry-ice for 1–2 hours and then in a 37° water-bath for 10 minutes. There followed five complete cycles of freezing (10 minutes) and thawing (10 minutes). After the final thaw 0.5 ml of 0.9 per cent NaCl was added containing 0.004 M magnesium ions and the enzyme DNase at 5 mg/ml (DNase II from Koch-Light Laboratories). Incubation was continued for 1 hour at which time it became possible to disperse the plug of DNA formed on rupture of the cells. The mixture was placed in boiled

(1/4 inch) dialysis tubing and dialysed against 19 ml of water for 16 hours at 4°. The dialysate was Millipore-filtered to sterilize and freeze-dried. The dried material was kept in sealed ampoules at -20°. On the day of use the contents of an ampoule were dissolved in Hanks' balanced salt solution (BSS) such that 100 μ l of solution contained extract from 3.5×10^6 nucleated cells.

Collection of peritoneal exudate cells

Macrophages were obtained in the form of a peritoneal exudate suspension. Hartley guinea-pigs aged 4-7 months received i.p. 25 ml of Bayol 85 (Esso). After 48 hours they were bled out from the heart under ether anaesthesia and the peritoneal cavity was washed out with approximately 100 ml of cold Hanks' BSS. The bulk of the oil was removed in a siliconed separating funnel and the cells were centrifuged at 220 g for 10 minutes at 4°. After washing, the cells from two animals of the same sex were combined and suspended in BSS to 10 per cent by volume.

Migration of peritoneal exudate cells

Migration of peritoneal exudate cells was carried out by the capillary tube technique (David and David, 1971). Capillaries of 1.0 mm diameter were centrifuged at 165 g for 2 minutes at 4°. Two capillary stubs containing the cell pellets were placed in each migration chamber together with 0.45 ml of medium. The chambers were sealed with glass cover slips and incubated at 37°. Media were gassed before use with 7 per cent O₂:10 per cent CO₂:83 per cent N₂ and incubation was carried out in the same atmosphere. After 20 hours the areas of migration were projected onto drawing paper and measured by planimetry.

The medium was RPMI 1640 (Flow Laboratories) containing 15 per cent normal guinea-pig serum that had been heated at 56° for 30 minutes. Penicillin and streptomycin were included in the medium at 50 units each per ml. PPD was used at 8 or 20 μ g/ml (purified protein derivative of human tuberculin, without preservative, was obtained from the Veterinary Laboratory, Ministry of Agriculture, Weybridge, Surrey). TF was used at a concentration of 100 μ l in 2.2 ml of medium.

Statistical analysis of migration areas

There were three chambers and thus six observations in each group. Square root transformations of the migration areas were analysed on a computer. The ratios of the root mean areas of particular groups together with 95 per cent and 99 per cent confidence limits were calculated by Fieller's theorem (Finney, 1952). Ratios quoted have been squared and multiplied by 100.

Ratios with non-overlapping limits at 95 per cent were taken as significantly different. Mean areas of migration were different from each other when the limits of their ratio did not take in the value of 100.

Correlation coefficients (Figs 2 and 3) and Student's *t*-tests (Figs 4 and 5) were calculated on the transformed data.

The sign test (Fig. 4) was used to assess the probability of a certain distribution of points being due to chance (Snedecor, 1967).

RESULTS

EFFECT OF PPD ON THE MIGRATION OF PERITONEAL EXUDATE CELLS

In all experiments controls were carried out to determine the effect of PPD in the absence of TF on the migration of peritoneal exudate cells from normal guinea-pigs. Areas of migration were determined at 20 hours and results have been expressed as the ratio of the mean area obtained in the presence of PPD to that obtained in its absence, multiplied by 100. This value has been termed 'antigen-control ratio'.

Fig. 1 shows the antigen-control ratios for each experiment. PPD has been used at 8 and 20 $\mu\text{g}/\text{ml}$. At the low concentration there were few values significantly different from 100, but at the high concentration such values formed about 40 per cent of the total. Both in-

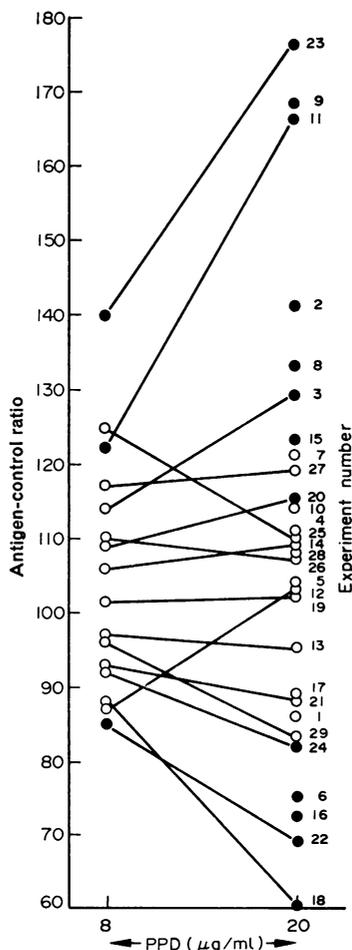


FIG. 1. Effect of PPD on the migration of peritoneal exudate cells from normal guinea-pigs. Antigen-control ratios are shown for each experiment. This is the ratio of the mean area of migration obtained in the presence of PPD to that obtained in its absence, multiplied by 100. Values are given for 8 and 20 $\mu\text{g}/\text{ml}$ of PPD and where both concentrations were used in the same experiment the two values are joined by a line. (●) Ratios significantly different from 100 at the 5 per cent or lower levels of probability. (○) Ratios not significantly different from 100.

hibitory and enhancing effects of PPD were seen. Where both concentrations were used in the same experiment the two ratios are connected by a line in Fig. 1, and it is seen that tendencies at low concentration were in most cases magnified at the high concentration. Apart from demonstrating the concentration dependence of these effects, this gives one confidence in the sensitivity and reliability of the technique.

EFFECT OF TF ON THE MIGRATION OF PERITONEAL EXUDATE CELLS

In all experiments the effect of the TF preparations on the migration of peritoneal exudate cells in the absence of PPD was determined. The ratio of the mean area obtained in the presence of TF to that obtained in its absence, multiplied by 100, was calculated

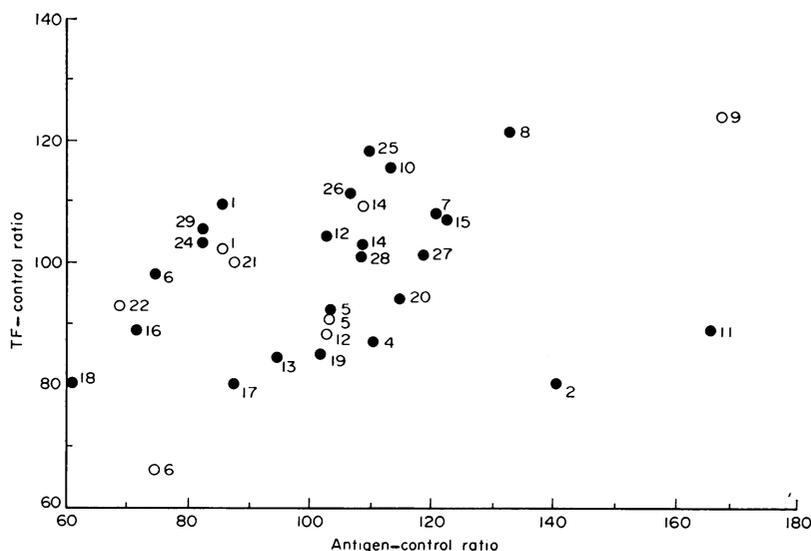


FIG. 2. Relationship between the effect of TF from tuberculin-positive donors and that of PPD on the migration of normal peritoneal exudate cells. Antigen-control ratios at 20 $\mu\text{g}/\text{ml}$ of PPD have been plotted against TF-control ratios for (●) donor JS and (○) other positive donors. The TF-control ratio is the ratio of the mean area obtained in the presence of TF to that obtained in its absence, multiplied by 100. The experiment number is shown with each point.

(TF-control ratio). There were few values significantly different from 100. The mean ratio (\pm s.d.) for TF from tuberculin-positive donors was 98.0 ± 13.3 (thirty-two observations) and for TF from negative donors was 94.1 ± 9.7 (eighteen observations).

An interesting correlation came to light when TF-control ratios (positive donors) were plotted against antigen-control ratios at the high level of PPD: higher values of TF-control ratio tended to occur at higher values of antigen-control ratio (Fig. 2). The correlation coefficient was calculated ($r = +0.381$) and confirmed a positive correlation at the 5 per cent level. There was no such correlation for negative TF (Fig. 3; $r = -0.214$), but the number of observations was smaller.

EFFECT OF TF IN THE PRESENCE OF PPD ON THE MIGRATION OF PERITONEAL EXUDATE CELLS

The effect of TF in the presence of PPD on the migration of peritoneal exudate cells has been analysed by comparing in each experiment the antigen-control ratio with the

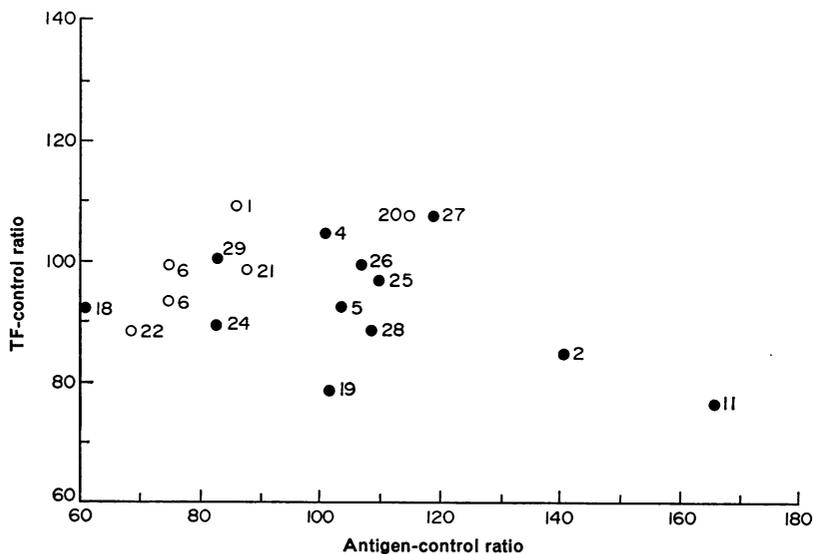


FIG. 3. Relationship between the effect of TF from tuberculin-negative donors and that of PPD on the migration of normal peritoneal exudate cells. Antigen-control ratios at 20 $\mu\text{g}/\text{ml}$ of PPD have been plotted against TF-control ratios for (●) donor DF and (○) other negative donors.

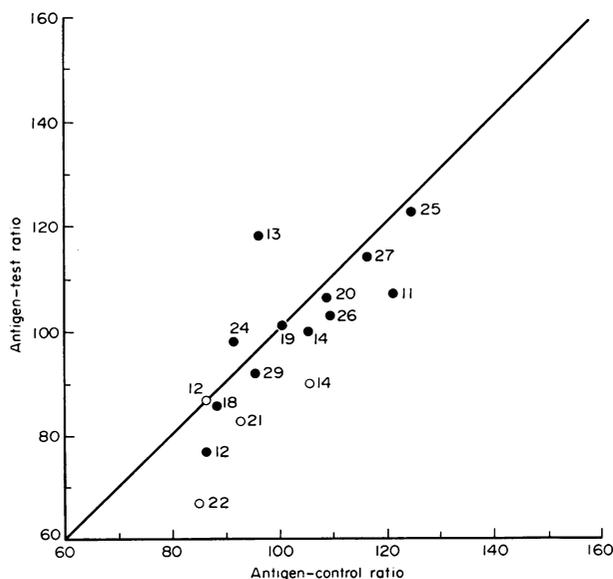


FIG. 4. Effect of TF from tuberculin-positive donors on the migration of normal peritoneal exudate cells in the presence of a low concentration of PPD. Antigen-control ratios at 8 $\mu\text{g}/\text{ml}$ of PPD have been plotted against the antigen-test ratios for (●) donor JS and (○) other positive donors. The antigen-test ratio is the ratio of the mean area obtained in the presence of TF and PPD to that obtained with TF alone, multiplied by 100. A line has been drawn through the origin with a gradient of 1.

antigen-test ratio. This latter value is the ratio of the mean area obtained in the presence of TF and PPD to that obtained in the presence of TF alone, multiplied by 100.

The antigen-control ratios at 8 $\mu\text{g}/\text{ml}$ PPD have been plotted against the corresponding antigen-test ratios. Fig. 4 shows the results with positive TF. If positive TF was doing nothing in the system that was antigen-dependent the points would be expected to fall

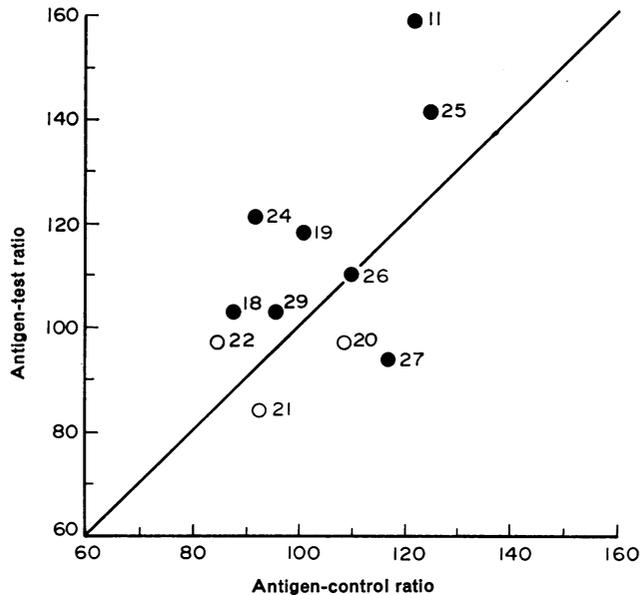


FIG. 5. Effect of TF from tuberculin-negative donors on the migration of normal peritoneal exudate cells in the presence of a low concentration of PPD. Antigen-control ratios at 8 $\mu\text{g}/\text{ml}$ of PPD have been plotted against the antigen-test ratios for (●) donor DF and (○) other negative donors. The antigen-test ratio is the ratio of the mean area obtained in the presence of TF and PPD to that obtained with TF alone, multiplied by 100. A line has been drawn through the origin with a gradient of 1.

TABLE I

EFFECT OF TF ON THE MIGRATION OF NORMAL PERITONEAL EXUDATE CELLS IN THE PRESENCE OF A HIGH CONCENTRATION OF PPD. EXPERIMENTS IN WHICH PPD ALONE HAD LITTLE EFFECT ON MIGRATION

Experiment	Antigen-control ratio	Antigen-test ratio	
		Positive TF	Negative TF
10	114	69 (JS)	—
4	111	26† (JS)	81 (DF)
5	104	64† (JS); 74* (DB)	90 (DF)
12	103	66† (JS); 106 (RO)	—
17	88	66 (JS)	—
1	86	62 (JS); 84 (AW)	96 (EC)

Experiments are shown in which the antigen-control ratio at 20 $\mu\text{g}/\text{ml}$ of PPD fell in the range 86–114 and the antigen-test ratio for one or more TF preparations was at least twenty units lower. Antigen-test ratios are given with TF donor in parentheses.

* Indicates an antigen-test ratio which is significantly different from the antigen-control ratio (* 5 per cent level). In experiments 4 and 5 the antigen-test ratio for JS is significantly lower than that for DF at the 1 per cent and 5 per cent levels respectively.

around the line that has been drawn through the origin with unit gradient. Although the points do follow the line quite closely, nearly all of them fall below. This unevenness in distribution of points can be analysed by the sign test. Twelve out of fourteen points fall below (points on the line are disregarded) and this represents a significant deviation from the line at the 1.3 per cent level. It seems therefore that there was a small inhibitory effect

TABLE 2
EFFECT OF TF ON THE MIGRATION OF NORMAL PERITONEAL EXUDATE CELLS IN THE PRESENCE OF A HIGH CONCENTRATION OF PPD. EXPERIMENTS IN WHICH PPD ALONE ENHANCED MIGRATION

Experiment	Antigen-control ratio	Antigen-test ratio	
		Positive TF	Negative TF
9	168 †	128 (AW)	—
11	166 †	150 (JS)	188 (DF)
2	141 *	217 (JS)	162 (DF)
8	133 *	96 (JS)	—
15	123 †	125 (JS)	—
7	121	132 (JS)	—
27	119 *	117 (JS)	97 (DF)
20	115 †	112 (JS)	93 (EC)

Experiments are shown in which the antigen-control ratio at 20 µg/ml of PPD was above 114.

* and † indicate an antigen-control ratio which is significantly higher than 100 (* 5 per cent level; † 1 per cent). None of the antigen-test ratios was significantly different from their antigen-control ratios.

TABLE 3
EFFECT OF TF ON THE MIGRATION OF NORMAL PERITONEAL EXUDATE CELLS IN THE PRESENCE OF A HIGH CONCENTRATION OF PPD. EXPERIMENTS IN WHICH PPD ALONE INHIBITED MIGRATION

Experiment	Antigen-control ratio	Antigen-test ratio	
		Positive TF	Negative TF
29	83	127 (JS)	89 (DF)
24	83 *	102 (JS)	92 (DF)
6	75 †	93 (JS); 91 (DB)	93 (DM); 88 (BM)
16	72 †	96* (JS)	—
22	69 †	103* (CT)	89 (EC)
18	60 †	86 (JS)	73 (DF)

Experiments are shown in which the antigen-control ratio at 20 µg/ml of PPD was below 86.

* and † indicate an antigen-control ratio significantly lower than 100 or an antigen-test ratio significantly different from its antigen-control ratio (* 5 per cent level; † 1 per cent).

of positive TF in the presence of antigen. On the other hand, the results with negative TF show no evidence of such inhibition (Fig. 5). Where both positive and negative TF were used in the same experiment the difference in the antigen-test ratios was calculated. Analysis of these differences by Student's *t*-test show that the values for positive TF are significantly lower ($P < 0.05$).

The picture obtained at 20 $\mu\text{g/ml}$ PPD is more complicated and the results are better shown in tabulated form. Table 1 shows a group of six experiments in which the antigen-control ratio is not significantly different from 100. In each case there was significant or apparent inhibition in the presence of TF from positive donor JS. One out of three other positive preparations also showed inhibition within this group. Negative TF where tested showed no effect or much less effect. However, there were seven other experiments with similar values of antigen-control ratio where both positive TF and negative TF (used in five of the experiments) had little effect (experiments 13, 14, 19, 21, 25, 26, 28).

Where antigen at 20 $\mu\text{g/ml}$ had an enhancing effect on migration in the control no significant differences were observed between antigen-test and antigen-control ratios for either positive or negative TF (Table 2). On the other hand, where antigen was inhibitory (Table 3) the effect was consistently overcome by TF. This action of TF was more pronounced with the positive material.

TABLE 4
EFFECT OF HEATED TF ON THE MIGRATION OF NORMAL PERITONEAL EXUDATE CELLS IN THE PRESENCE OF PPD (20 $\mu\text{g/ml}$)

Experiment	Antigen-control ratio	Antigen-test ratio			
		Positive TF		Negative TF	
		Normal	Heated	Normal	Heated
5	104	64 (JS)	122† (JS)	90 (DF)	102 (DF)
		74 (DB)	143* (DB)	—	—
17	88	66 (JS)	88 (JS)	—	—
6	75	93 (JS)	75 (JS)	93 (DM)	67 (DM)
		91 (DB)	103 (DB)	88 (BM)	79 (BM)

TF was used either before or after heating at 56° for 30 minutes.

* and † indicate a significant difference between the antigen-test ratios for normal and heated TF (* 5 per cent level; † 1 per cent).

EFFECT OF HEAT ON THE ACTIVITY OF TF

Lawrence and Zweiman (1968) reported that heating TF for 30 minutes at 56° destroys activity *in vivo*. This has not been routinely tested in the present *in vitro* experiments, but it has been found that effects of TF in the presence of antigen are generally abolished by this procedure. Three examples are shown in Table 4.

DISCUSSION

Two notable features of the transfer factor phenomenon are the rapidity with which sensitivity appears in the recipient (within 18 hours) and its long duration over months or years (Lawrence 1969a,b). Both these observations may be explained in terms of an antibody-like structure for TF.

Let us postulate that following injection TF binds to blood macrophages. This process is rapid, thus explaining the rapidity of sensitization. On skin-testing, the now 'receptored' macrophage finds the antigen and a delayed response ensues. This comes about either

because the macrophage is activated to produce the necessary mediators or because antigen, bound to macrophages in this way, immunizes specific thymus-derived (T) lymphocytes so rapidly that their mediators are the effective agents. Whichever mechanism applies in this first delayed reaction, the model suggests that subsequent reactions are dependent on the presence of sensitive T cells. Normally skin-testing does not immunize, but in the presence of TF immunization does occur and that could be the explanation of the long duration of transferred sensitivity.

If TF is antibody-like, a logical suggestion would be that it is a T-cell product related or identical to the receptor. Relevant to this could be the finding of Lawrence and Pappenheimer (1956, 1957) that TF disappears from sensitive cells after a short incubation with antigen. Macrophages are thus implicated in our hypothesis by analogy with their binding of certain antibody-like factors probably of T-cell origin (Amos and Lachmann, 1970; Feldmann, 1972; Evans, Grant, Cox, Steele and Alexander, 1972). Although the mediators of delayed hypersensitivity are generally thought to be produced by T cells, the possibility that macrophages are a source has not been excluded (Gery and Waksman, 1972; Havemann, Burger, Schmidt, Sodomann and Stein, 1973). On the other hand, macrophage-bound antigen is known to be a potent stimulator of T cells (Unanue and Feldmann, 1971; Parish, 1972; Seeger and Oppenheim, 1972), and it could be that if the binding is through a specific T-cell product induction will be particularly efficient (Feldmann and Nossal, 1972).

A difficulty in this concept of TF is that of molecular size. If we accept that TF possessing specificity is dialysable, its molecular weight is unlikely to exceed 20,000. Moreover, there is some preliminary evidence suggesting a value of less than 10,000 (Lawrence, 1969a). The minimum size consistent with an antibody-like structure would be 10,000, equivalent to one variable region, unless one is to invoke the possibility of a smaller combining region perhaps held in the native configuration by linkage to RNA, as may occur with antigenic fragments (Gottlieb and Schwartz, 1972). The RNA might also provide the cytophilic property.

How far do the present results substantiate the hypothesis? Two pieces of evidence suggest a similarity between TF and antigen-dependent MIF. First, TF from donors sensitive to PPD was found to have a small but consistent inhibitory effect on the migration of peritoneal exudate cells from normal guinea-pigs in the presence of a low concentration of PPD (8 $\mu\text{g}/\text{ml}$). Such inhibition was not seen using negative TF. The second point concerns experiments at the high concentration of PPD (20 $\mu\text{g}/\text{ml}$). In about half those experiments where this level of PPD had little effect on the control migration, strong inhibition occurred in the further presence of positive TF. No explanation can be given for this lack of consistency, but the inhibition was not seen with negative TF.

Where PPD (20 $\mu\text{g}/\text{ml}$) had a marked enhancing effect on migration no significant modification was observed in the further presence of TF, but where PPD was inhibitory a consistent counteraction occurred. Although this applied to both positive and negative TF, positive TF was more potent. A similar counteraction did not occur at the low concentration of PPD (Fig. 4) and its significance is obscure. The inhibition seen with PPD alone is presumably due to naturally acquired immunity to PPD or a cross-reacting antigen. Enhancement, also reported by Hay, Lachmann and Trnka (1973), is difficult to explain. A similar phenomenon occurs in the human buffy-coat migration system (Soburg, 1968; Federlin, Maini, Russell and Dumonde, 1971).

A correlation was thus observed between the activity of TF as an antigen-dependent

MIF and the sensitivity of TF donor. But there are a number of important qualifications. Firstly, the bulk of the data was obtained with one positive and one negative donor, and only one antigen was investigated. Secondly, this correlation could not establish unequivocally an antibody-like structure for TF. Peritoneal exudates contain some lymphocytes. TF, whatever its nature, could be transferring sensitivity to these so that they produce MIF in the presence of antigen (David, 1971). It will be important therefore to test purified macrophages. Finally, in these experiments the term TF has been used to describe a preparation rather than an activity since the material has not been tested by injection in man. The preparations are likely to contain TF in view of the reproducibility of the phenomenon in many laboratories (Lawrence, 1969a), and the experience with the heated preparations suggests that TF is being assayed. Nevertheless, until tested directly it must remain an assumption that an antigen-dependent material is our dialysed preparations is in fact TF.

A mechanism involving direct action of TF on lymphocytes would relate the present system to that of Paque, Dray, Kniskern and Baram (1973), who treated human non-sensitive lymphocytes with undialysed extracts of sensitive cells and the homologous antigen and showed that MIF was produced. Unfortunately, no effect was observed with the dialysate. Ascher, Schneider, Valentine and Lawrence (1973) have tested the dialysate with promising results in a similar system involving determination of DNA synthesis. A routine assay based on the present system is clearly some way off but it remains a definite possibility.

If TF should turn out not to have an antibody-like structure, antigen seems a more likely candidate to provide the specificity than informational nucleic acid. Furthermore, the data in Fig. 2 showing a correlation between the effects of PPD on migration and those of positive TF support the view that TF is a form of antigen. This would be a satisfactory solution from the point of view of molecular weight but there are a number of problems. Induction of sensitivity occurs much too rapidly for a normal antigen, and a 'super-antigen' should be easy to demonstrate. However, to my knowledge there are no reports of delayed reactions at the site of injection of the dialysate and neither Lawrence (personal communication) nor I have been able to demonstrate transformation of lymphocytes from a positive individual by TF alone. Nevertheless, antigen could be present in the buffy-coat cells used for preparation of TF. It could be there, perhaps in monocytes, as a result of (a) the initial sensitization, (b) the presence of antigen in the environment, or (c) the skin-test that is normally applied. Points (b) and (c) may only operate if sensitization has already occurred, thus maintaining specificity of TF. One possibility is that TF is RNA linked to an antigenic fragment, and a number of studies have appeared showing a TF-like activity in RNA extracts of sensitive cells (Paque and Dray, 1972).

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