Immunology, 1963, **6**, 484.

A Factor Capable of Increasing Vascular Permeability Present in Lymph Node Cells

A POSSIBLE MEDIATOR OF THE DELAYED REACTION

D. A. WILLOUGHBY, BARBARA BOUGHTON AND H. O. SCHILD

Department of Pathology, St. Bartholomew's Hospital, London and Department of Pharmacology, University College, London

(*Received 26th February* 1963)

Summary. A soluble extract from guinea-pig lymph node cells (LPF) has been found to increase vascular permeability in the skin of the rat. The active substance has been differentiated from histamine, 5-hydroxytryptamine, bradykinin, substance P, kallikrein and the globulin permeability factors from rat and guinea-pig serum by means of parallel quantitative assays. LPF was present in both sensitized and non-sensitized guinea-pig lymph node cells and in lymph node cells from rats and mice. LPF also increased vascular permeability in the skin of guinea-pigs, mice and rabbits. The possible importance of this factor in the mechanism of the delayed reactions is discussed.

INTRODUCTION

A great deal is known about histamine and other mediators released during the anaphylactic reaction but relatively little about the chain of events leading to the delayed or tuberculin-type hypersensitivity reaction.

A number of attempts have been made to identify the substances involved and one line of investigation suggests that histamine may be a mediator. It has been shown that the histamine content of the skin at the site of a delayed reaction rises. In a tuberculin reaction the increase was found to parallel the development of the lesion and was greater than could be accounted for by infiltration of mononuclear cells (Inderbitzin, 1961). Similarly, in the allergic reaction to dinitrochlorobenzene the histamine content of the lesion is greater than that in the primary toxic response to this chemical (Fisher and Cooke, 1958). The change could be brought about by reduced destruction or increased production of histamine and, although there is no evidence for the former, Schayer and Ganley (1959) have reported that histidine decarboxylase is increased in the tuberculin reaction. This led Schayer (1959) to suggest that the lesions are due to the exposure of tissue to a continuous slow release of histamine. However, Fisher and Cooke (1958) found that lesions lasted longer in animals treated with a decarboxylase inhibitor and assumed that histamine was involved in the repair process.

The second approach to this probelm was the study of substances released during *in vitro* incubation of sensitized cells with antigen. This yielded very little result until Johanovsky (1959) demonstrated that a pyrogenic substance could be released specifically from

sensitized cells in the presence of antigen. In later work Johanovsky (1961) showed that this pyrogen was rapidly formed when antigen was added to an extract from sensitized cells, and that its *in vitro* formation could be inhibited by protease inhibitors (Johanovsky and Skvaril, 1962).

In a previous communication (Willoughby, Boughton, Spector and Schild, 1962) we reported the presence in guinea-pig lymph node cells of an active principle which increases vascular permeability. This factor could be differentiated from other known permeability factors by means of parallel quantitative assays. These preliminary observations have now been extended and confirmed.

EXPERIMENTAL METHODS

Measurement of Vascular Permeability

Rats anaesthetized with ether were injected intravenously with 0·4 ml. per 100 g. body weight trypan blue (1 per cent w/v in isotonic saline) followed by intradermal injection of 0·1 ml. of test solution. The rats were killed 30 minutes later, their skin stripped off, and readings of the degree of blueing of the inner surface of the skin were taken either by inspection or by a colorimetric method based on the extraction of the dye from the injection site (Judah and Willoughby, 1962). Similar methods were used for mice.

For tests carried out on guinea-pigs the unanaesthetized animals were injected intravenously into an ear vein with 1·2 ml./kg. pontamine blue (5 per cent in isotonic saline); 0·1 ml. of the test substances was introduced intradermally into the shaved flank. The mean of the two diameters measured at right angles of the blue lesion on the outer surface of the skin was taken as an index of blueing. A similar method was used for rabbits. To test the effect of mepyramine maleate the permeability factors were injected into the same guinea-pig before and after intravenous administration of 0·1 mg./kg. mepyramine maleate.

Blood Pressure

This was measured by a mercury manometer in dogs, cats, rabbits and guinea-pigs under pentobarbitone anaesthesia and in rats under urethane anaesthesia.

Guinea-Pig Ileum Preparation

A segment (1–2 cm.) of terminal ileum was suspended in oxygenated Tyrode solution in a 1 ml. bath at 38°.

Histamine was assayed using an automatic apparatus (Boura, Mongar and Schild, 1954). Absence of bradykinin-like activity of the cell extracts was demonstrated by leaving them in contact for periods of up to 3 minutes with ileum which gave a strong contraction to 50 ng./ml. bradykinin (Sandoz) in the presence of 10^{-9} mepyramine.

Kinin formation from heated (56°, 3 hours) dialysed dog plasma was detected by its contractile activity on the ileum in the presence of 10^{-8} atropine and 10^{-8} mepyramine. 0·2 ml. of dog plasma was added to a 1 ml. bath followed immediately by 0·1 ml. of the test preparation. Contact was allowed for 1 minute. Fresh heated, dialysed guinea-pig plasma was also used as a substrate.

Rat Uterus

A horn of uterus from a virgin rat was suspended in de Jalon solution in a 5 ml. bath; 10^{-6} dibromolysergic acid diethylamide (BOL 148) was used in the washing fluid to antagonize 5-hydroxytryptamine.

Histamine Release

The ability of test solutions to release histamine from chopped guinea-pig lung was tested by the method of Mongar and Schild (1957) using 2 ml. samples and a 30 minute incubation period.

Dialysis

This was carried out at 4° in Visking tubing against three changes of saline or water. No histamine- or 5-hydroxytryptamine-like activity could be detected after this treatment in the material present in the dialysis sac when tested on guinea-pig ileum and rat uterus.

Preparation of Lymph Node Cell Permeability Factor (LPF)

Guinea-pigs were sensitized to tuberculin by intradermal injection of an emulsion consisting of equal volumes of Freund's Incomplete Adjuvant and a saline suspension of steam-killed human tubercle bacilli (15 mg. dry wt./ml.). A total volume of 0·4 ml. was injected at multiple sites in the back of the neck and 0·1 ml. into each hind foot. In the first instance, for comparisons of sensitized and normal cells, only the enlarged nodes draining the injection sites were taken from sensitized animals whilst all possible nodes were taken from uninjected pigs. In later experiments in which only sensitized animals were used all nodes of a reasonable size were taken.

The lymph nodes were freed of fat and the cells teased out into Tyrode solution between two pairs of forceps. The suspension was freed of cell debris by passage through fine nylon and the cells collected by centrifugation in an MSE angle centrifuge at 4000 rev./min. for 40 seconds. The pad of cells was washed twice by resuspension in Tyrode solution and the number of cells estimated using a Burker counting chamber. The final suspension contained 500 million cells per ml. and was treated in one of the following ways:

Ultrasonic disintegration. After treating cells with an MSE disintegrator at 20,000 cycles/sec. for 1½ minutes, microscopic examination revealed no intact cells but only debris which was removed by centrifugation at 4000 rev./min. for 15 minutes. Most of the experiments were carried out with the slightly opalescent supernatant obtained in this way.

Freeze thawing. Cell suspensions were frozen and thawed approximately ten times until microscopic smears showed no whole cells. The supernatant obtained in this way had approximately the same activity as that obtained by ultrasonic disintegration.

Other Materials

Salivary kallikrein was prepared by acetone precipitation from human saliva (Holdstock, Mathias and Schachter, 1957). The final aqueous extract was stored in the frozen state and represented a five-fold concentrate of the original saliva. A sample of substance P, (50 units/mg.) was obtained from Dr. J. H. Gaddum. Two samples of bradykinin were used: a synthetic preparation from Sandoz Ltd. and a sample obtained from Dr. G. P. Lewis (P2-fraction) which had an activity of one four-hundredth pure bradykinin (Sandoz).

Guinea-pig globulin permeability factor (Wilhelm, Miles and Mackay, 1955) and rat globulin permeability factor were prepared by the method described by Spector (1956) which consists of ether/ethanol fractionation of the respective sera. The term 'standard globulin preparation' refers to the fractionated serum which has been reconstituted to the original volume. Diisopropylfluorophosphonate was obtained as a 1 per cent solution in arachis oil from Boots Pure Drug Co. Soya bean trypsin inhibitor was obtained from L. Light and Co.

Padutin from Bayer Products Ltd. contained the extracted matter of 7 g. pancreas glands per ml.

Other substances used were standard commercial preparations.

RESULTS

PERMEABILITY EFFECTS

Unless otherwise mentioned the following experiments refer to a factor in cell-free extracts obtained by ultrasonic treatment from lymph node cells of tuberculin-sensitized guinea-pigs and tested in rats for ability to increase vascular permeability. This will be referred to as LPF.

Effect of LPF on Rat Skin

Undialysed LPF produced a marked increase in vascular permeability in rat skin in doses corresponding to 1·5–25 million cells (approximately 0·75–12·5 mg. wet wt. of cells). As shown in Fig. 1 the extract produced a graded response by which its activity could be

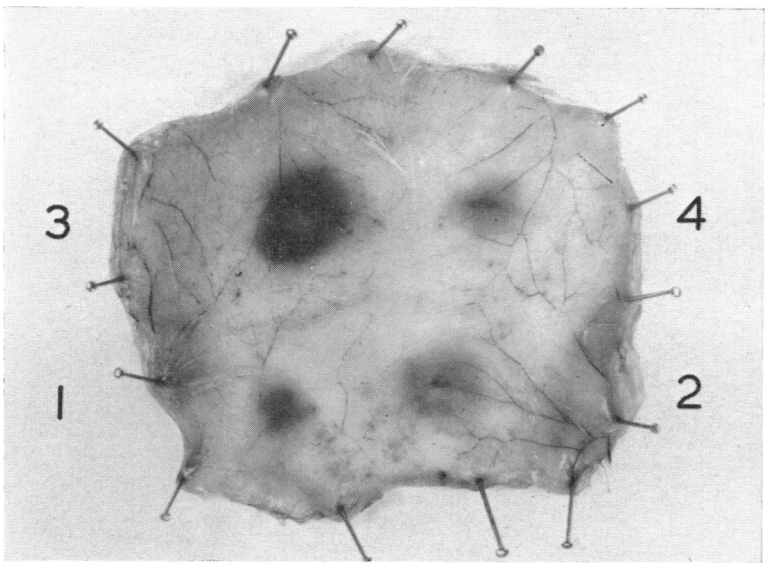

FIG. 1. Areas of blueing on the inner surface of the skin of a rat which had received trypan blue (4 mg. per 100 g.) intravenously followed by intradermal injections of 0·1 ml. of extracts corresponding to (1) 5, (2) 10, (3) 20 million guinea-pig lymph node cells and (4) kallikrein from 0·5 ml. of human saliva.

assayed in terms of other permeability factors. The effect of extracts from 10 million cells was equivalent in activity to that produced by the following: kallikrein derived from 1 ml. of human saliva, bradykinin (P2) 10 μg., histamine 10 μg., hydroxytryptamine 1 μg., substance P 0·1 unit and 0·05 ml. of the standard preparation of globulin PF from rat or guinea-pig serum.

Release of a Permeability Factor from Guinea-Pig Lymph Node Cells by Standing

Incubation of viable lymph node cells at 37° for periods of up to 8 hours gave rise to a slow release of this factor from the cells. The activity released in this way was less than that

of the supernatant from cells which had been completely disrupted by freeze-thawing or ultrasonic disintegration.

Presence of a Permeability Factor in Lymph Node Cells of Animals other than Sensitized Guinea-Pigs

Extracts of cells from the lymph nodes of normal guinea-pigs were found to have a similar activity on rat skin as extracts of an equivalent number of cells from the nodes of sensitized animals. Extracts of lymph node cells from mice and rats also had similar activity and gave similar dose response relations.

THE EFFECT OF VARIOUS TREATMENTS AND ANTAGONISTS ON THE ACTIVITY OF LPF

Overnight dialysis caused no appreciable loss of activity of LPF.

Pretreatment of rats with mepyramine maleate (1 mg./kg.) or BOL 148 (2 mg./kg.) had no effect on the blueing activity of LPF. However, these doses of antagonist were sufficient to abolish the effect of equiactive doses of histamine and 5-HT respectively.

Pretreatment of rats with 3 mg./kg. diisopropylfluorophosphonate (DFP, 1 per cent in arachis oil) failed to modify the permeability changes induced by LPF obtained from 1·25–5 million cells but caused a striking reduction in the permeability changes brought about by rat or guinea-pig globulin PF.

Incubation of LPF with soya bean trypsin inhibitor (100 μg.) for 20 minutes failed to modify its vascular effect.

When LPF was mixed with guinea-pig plasma and injected 3 minutes later its effect on

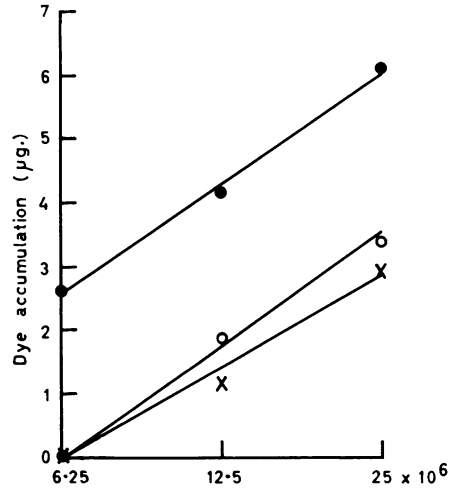

FIG. 2. Dye accumulation at the site of intradermal injection in rats of 0·1 ml. of an extract of guinea-pig lymph node cells alone (●), and together with sodium salicylate (○) and guinea-pig plasma (×).

vascular permeability was markedly reduced as shown in Fig. 2. A similar reduction of activity occurred when 2·5 mg. of sodium salicylate was mixed with LPF before injection (Fig. 2).

THE EFFECT OF LPF ON VASCULAR PERMEABILITY OF OTHER SPECIES

Extracts of lymph node cells from guinea-pigs were also active in increasing vascular permeability in the skin of mice, rabbits and guinea-pigs. Certain differences, however, existed in the vascular responses of these other species.

The mouse gave a very good response to LPF which was similar to that obtained in the rat in size of lesion and intensity of dye leakage measured colorimetrically (Fig. 3.) The effect on mouse skin, however, was reduced after dialysis of LPF.

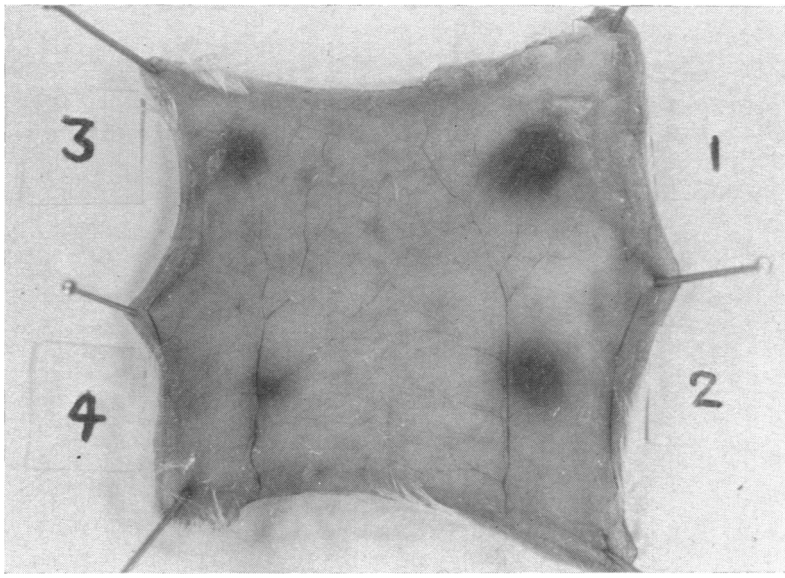

FIG. 3. Dye accumulation on the inner surface of the skin of a mouse at the site of intradermal injections of 0·05 ml. amounts of LPF corresponding to (1) 12·5, (2) 6·25, (3) 3·1 and (4) 1·5 million guinea-pig lymph node cells.

The rabbit was the least responsive of these species to LPF but a weak response could be obtained with extracts of 25 million cells.

The effect of LPF on vascular permeability in the guinea-pig was more variable than in the rat. Out of nine separate preparations, each of which was tested in at least two different guinea-pigs, six gave good responses, extracts equivalent to 2 and 3 million cells giving lesions greater than those due to saline alone (Table 1). However, there was some variation in the sensitivity of individual animals. Two of the nine preparations were active only in doses equivalent to 50 and 25 million cells (Preparations a and f—Table 1) whilst a third gave an effect only with a dose equivalent to 50 million cells in one animal but gave no effect with 25 in a second animal (Preparation h—Table 1). Table 2 shows the relation between number of cell equivalents injected and reaction size in guinea-pig skin. It is obvious that similar dose response relationships are obtained with normal and with sensitized cells.

Overnight dialysis of three preparations of LPF did not appreciably affect their permeability activity on guinea-pig skin (Table 3) which suggests that the effects are not

due to an admixture of histamine. Furthermore, the dose of histamine required to parallel the effect of LPF (Fig. 4) was 10–50 times that present in the undialysed extract. Nevertheless, the effects of LPF on guinea-pig skin were markedly reduced by pretreatment of the animal with mepyramine maleate (0·1 mg./kg.) as shown in Fig. 4. This contrasts with the effect of LPF in rat skin which is unaffected by mepyramine.

TABLE 1

EFFECT OF INTRADERMAL INJECTIONS OF LPF (0·1 ml.) INTO GUINEA-PIGS PREVIOUSLY TREATED WITH PONTAMINE BLUE (60 mg./kg.)

LPF preparation	*Guinea-pig*	*Activity of LPF*	
		Diameter (mm.) of blue lesions produced by 50×10^6 cell equivalents	*Minimum active dose (million cell equivalents)*
a	1	5	50
	2	6	50
b	3	5, 6 (5·5)	10
	4	9, 8, 7, 4 (7·4)	Not tested
c	5	9	2
	6	9	2
d	7	8, 8 (8)	2
	8	6	10
	9	8, 9 (8·5)	3
	10	8, 8 (8)	<12·5
e	11	10	<6
	12	10	<3
f	13	4	50
	14	6	25
g	15	10	3
	16	10	0·4
	17	9	2
h	18	5	50
	19	Not tested	>25
k	20	10	0·4
	21	9	2
	22	5	10

DIFFERENTIATION FROM OTHER PERMEABILITY FACTORS

By means of antagonists and parallel quantitative assays the effects of LPF could be distinguished from those of a variety of other permeability factors. The indices of discrimination (Gaddum, 1955) obtained are summarized in Table 4. LPF was assayed against each factor separately on rat skin and then used for comparative assays on other preparations.

TABLE 2

COMPARISON OF THE EFFECTS OF LPF FROM SENSITIZED (S) AND NON-SENSITIZED (N) ANIMALS ON THE SKIN OF GUINEA-PIGS PREVIOUSLY INJECTED INTRAVENOUSLY WITH PONTAMINE BLUE (60 mg./kg.)

Extracts compared	*No. of cell equivalents injected (million)*	*Diameter of blue lesion (mm.)*	
		Sensitized	*Non-sensitized*
S and N1	50	10	10
	25	8	8
	12·5	7	7
	6·25	6	6
	3·1	4	6
S and N2	50	10	9
	10	8	7
	2	6	5
	0·4	5	5
	50	9	6
	10	6	4
	2	4	3
	0·4	3	3

TABLE 3

EFFECT OF OVERNIGHT DIALYSIS OF LPF ON ITS PERMEABILITY-INCREASING ACTIVITY IN THE SKIN OF GUINEA-PIGS PREVIOUSLY INJECTED INTRAVENOUSLY WITH PONTAMINE BLUE (60 mg./kg.)

LPF preparation	*No. of cell equivalents injected (million)*	*Diameter of blue lesion (mm.)*		
		Untreated	*Dialysed*	
e	50	10	8	
	25	10	6	
	12·5	8	5	
	6·25	6	4	
	3·1	5	2	
	50	10	8	
	25	10	8	
	12·5	9	7	
	6·25	8	6	
f	50	6	8	8
	25	4	7	7
	12·5	2	6	5
	6·25	3	5	3
k	50	10	8	8
	10	9	7	8
	2	7	4	5
	0·4	4	2	3

Histamine and 5-Hydroxytryptamine

Undialysed cell-free extracts of guinea-pig lymph node cells contain histamine-like and 5-HT-like activity which is lost after overnight dialysis against saline. Undialysed extracts produced a contraction of rat uterus corresponding to a concentration of 1–10 ng. of 5-HT

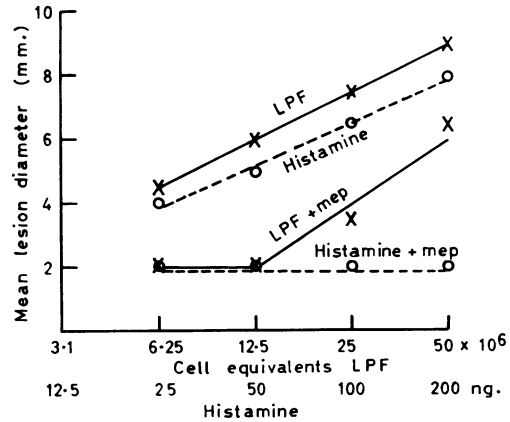

FIG. 4. Diameter of the blue lesion produced by intradermal injection of 0·1 ml. of LPF (×) and histamine (○) in the skin of a guinea-pig which had previously received pontamine blue (60 mg./kg.). Size of lesions before and after treatment of the animal with mepyramine maleate (0·1 mg./kg.).

TABLE 4

COMPARISON OF THE EFFECT OF LPF AND OTHER PERMEABILITY FACTORS ON RAT VASCULAR PERMEABILITY AND ON OTHER TEST PREPARATIONS

Discrimination from	*Preparation*	*Index of discrimination*	*Other points of difference*
Histamine	Guinea-pig ileum	2500	LPF unaffected by mepyramine LPF non-dialysable
5-Hydroxytryptamine	Rat uterus	1000	LPF non-dialysable LPF unaffected by BOL 148
Bradykinin			
P2 fraction	Rat uterus	>1000	LPF inhibited by salicylate which does not affect bradykinin or substance P
Synthetic	Guinea-pig ileum	>10000	
	Dog blood pressure	>500	
Substance P	Rabbit blood pressure	>20	
Kallikrein			
Salivary	Dog blood pressure	>800	Kallikrein inhibited by SBTI which had no effect on LPF
	Kinin formation	800	
Padutin	Dog blood pressure	>400	
Globulin PF			
Guinea-pig	Guinea-pig blood pressure	>80	Inhibited by SBTI and DFP which do not affect LPF
	Guinea-pig skin	200	
Rat	Guinea-pig skin	80	

per 10 million cells which could be abolished by addition of BOL 148. The extracts also caused a contraction of guinea-pig ileum corresponding to 1–5 ng. histamine per 10 million cells. This effect was abolished in the presence of mepyramine maleate.

As has already been recorded, the effect on vascular permeability of rat skin was unaffected by pretreatment of the animal with either of these inhibitors.

Bradykinin

The distinction of LPF from bradykinin is based on the effects of this polypeptide on guinea-pig ileum, rat uterus and dog blood pressure. A dialysed sample of LPF from 500 million cells had no effect on a guinea-pig ileum preparation which responded to 50 ng. of synthetic bradykinin and a rat uterus preparation which responded to 500 ng. of bradykinin (P2 fraction).

A blood pressure preparation of the dog which responded with a fall of 20 mm. Hg to a dose of 4 μg. of synthetic bradykinin and exhibited a distinct slowing of the heart rate after 50 μg. of bradykinin (P2) was completely unaffected by two separate doses of LPF each corresponding to 2000 million cells (Fig. 5a).

A further discrimination was provided by salicylate which, as already mentioned, inhibits the permeability effect of LPF when injected in a dose of 2·5 mg. together with LPF but fails to inhibit bradykinin under similar conditions.

Substance P

In a blood pressure preparation of the rabbit in which 0·5 units of substance P caused a fall of blood pressure of 14 mm. Hg an LPF extract from 500 million cells had no effect (Fig. 5b). A further point of difference is that the permeability activity of this polypeptide is unaffected by salicylate.

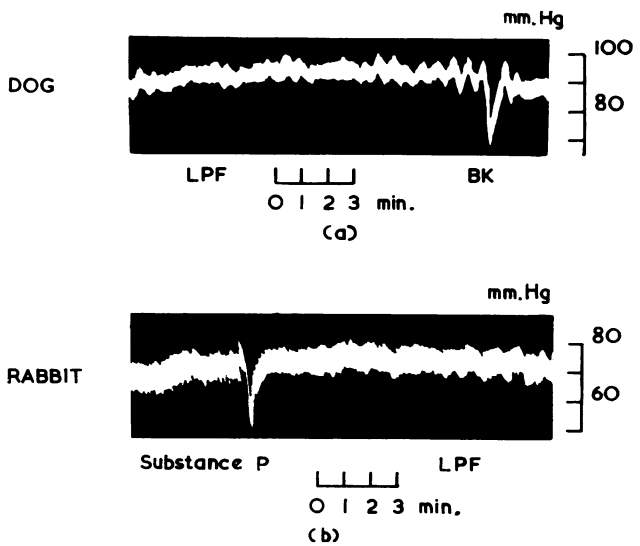

FIG. 5. Effect in an 11 kg. dog of intravenous injections of pure bradykinin (BK)—4 μg. and of LPF extracted from 2000 million lymph node cells. (b) Comparison of the effects of intravenous injection of substance P (0·5 unit) and LPF extracted from 500 million cells in a 3 kg. rabbit.

Kallikrein

The preparation of salivary kallikrein used produced a powerful depression of a dog's blood pressure: an amount equivalent to 0·5 ml. of human saliva producing a fall of 20 mm. Hg. A commercial sample of Padutin also lowered the dog's blood pressure in a dose

of 0·5 ml. whereas LPF extracted from 2000 million cells had no effect (Fig. 6). The resulting indices of discrimination were >800 and >400 for salivary kallikrein and Padutin respectively.

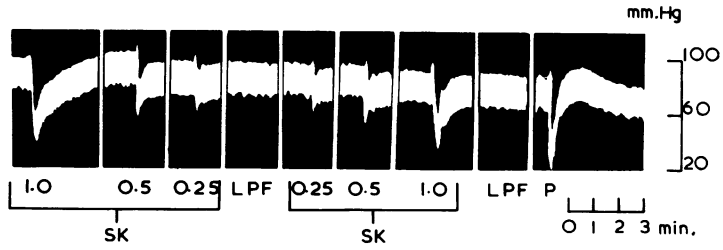

FIG. 6. Effect in a 6·5 kg. bitch of intravenous injections of human salivary kallikrein (SK) equivalent to 0·25, 0·5 and 1·0 ml. of human saliva and 0·5 ml. of Padutin (P) compared with the effect of two injections of LPF each extracted from 2000 million cells. This dose of LPF was equivalent to kallikrein from 200 ml. saliva and to 200 ml. Padutin when assayed on rat skin.

The salivary kallikrein preparation formed kinins when incubated with heated, dialysed dog plasma or similarly treated guinea-pig plasma. An amount corresponding to 0·06 ml. of saliva was active in this respect whereas extracts of up to 500 million cells were inactive, giving an index of discrimination of 800. The permeability effects of kallikrein and LPF could also be differentiated by treatment with soya bean trypsin inhibitor (SBTI). Incubation with 100 μg. of SBTI inhibited the effect of kallikrein but had no effect on that of LPF.

Globulin Permeability Factors from Rat and Guinea-Pig Serum

Guinea-pig blood pressure preparation was used to distinguish the globulin permeability factors from rat and guinea-pig serum, as prepared by the method of Spector (1956), from LPF. Guinea-pig globulin PF had a powerful hypotensive effect in this preparation confirming the results of Wilhelm *et al.* (1955). A fall of 10–15 mm. Hg was brought about by a dose corresponding to 0·0025 ml. of original serum. The response was biphasic and graded according to dose. When doses were injected at 3 minute intervals there was no tachyphylaxis. Preparations of rat globulin PF had a hypotensive effect which exhibited strong tachyphylaxis. After the first injection a much higher dose was required to produce a response and it was thus not possible to establish a dose–response curve. The effect of LPF on this preparation was variable and appeared to depend on storage, as three freshly prepared samples of LPF were not active. The response to two stored preparations of LPF was dependent on dose and considerably less than that of equiactive skin doses of guinea-pig globulin PF and exhibited no tachyphylaxis. In Fig. 7 the effect of guinea-pig globulin

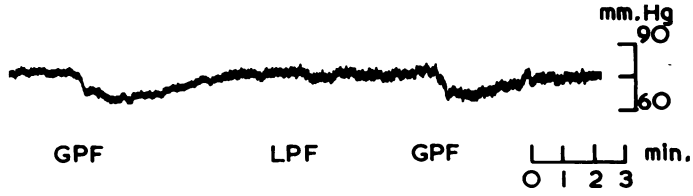

FIG. 7. Comparison of the effects of intravenous injection of guinea-pig globulin PF (GPF) from 0·0025 ml. of serum and LPF from 250 million cells in a 500 g. guinea-pig. This dose of LPF has 80 times the activity of the dose of globulin PF used when assayed on rat skin.

PF is compared with that of a freshly prepared sample of LPF on guinea-pig blood pressure, and an index of discrimination of 80 was obtained.

Guinea-pig skin in parallel quantitative assays with rat skin gave the best discrimination between LPF and the globulin factors. Preparations of LPF, guinea-pig globulin PF and rat globulin PF which had equal permeability activity in rat skin gave equivalent responses in guinea-pig skin when used in dilutions of 1 : 200 : 80. The globulin permeability factors gave clearly defined bright blue reaction areas as opposed to the more diffuse lesions due to LPF.

A further distinction between guinea-pig globulin PF and LPF is provided by incubation with SBTI which inhibits the permeability activity of the serum globulin factor but has no effect on that of LPF. Furthermore, pretreatment of the rat with DFP also inhibits globulin PF but leaves LPF unaffected.

OTHER PROPERTIES OF LPF

The permeability increasing activity of the cell extracts on rat skin was unaffected by dialysis at 4°, indicating that this activity is associated with a large molecule.

In an attempt to purify the extract saturated ammonium sulphate solution was added in the cold until a precipitate formed (66 per cent saturation). This on separation, reconstitution and dialysis to remove the ammonium sulphate had about 50 per cent of the activity of the original extract.

An extract which had been dialysed overnight against water and then freeze-dried showed no loss of activity on reconstitution in saline despite the presence of a pale brownish insoluble residue. The dry weight of a dialysed extract obtained from 500 million cells was 14 mg.

An extract from cells which had been stored at 4° for 4 weeks before disruption and a cell-free extract treated similarly showed no loss of activity on rat skin compared with a freshly prepared extract.

The activity was undiminished in cell-free extracts which had been heated at 37° or 56° for 30 minutes, but the activity was reduced when incubation at 37° was continued for 2 hours.

Histamine-Releasing Activity

It was thought that the effects obtained on guinea-pig skin may be due to histamine-releasing activity of the cell extract. Incubation of guinea-pig lung with amounts of the extract corresponding to 20,000 to 500 million cells gave no histamine release above the control value.

DISCUSSION

Although the exact role of the circulating mononuclear cells in delayed hypersensitivity reactions is unknown there seems to be little doubt that in some way they participate in the inflammatory vascular response which is characteristic of tuberculin-type reactions. The present series of experiments have shown that lymph node cells contain a permeability factor which may be regarded as a potential mediator of the inflammatory reaction.

This permeability factor, which has been termed 'LPF', can be distinguished from other permeability factors. It is readily distinguished from the small molecular compounds such as histamine and 5-hydroxytryptamine by its inability to move through dialysis tubing, by its lack of activity on smooth muscle preparations and finally by the use of specific an-

tagonists such as mepyramine maleate and BOL 148. No evidence could be found to suggest that LPF was acting as a kinin-forming enzyme nor, in the rat, as a histamine liberator. On the other hand in guinea-pig skin histamine release may contribute to the response, as pretreatment of the animal with small doses of mepyramine reduced the effect. However, direct measurement using isolated lung particles showed no evidence of histamine-releasing activity.

Parallel assays were made on a variety of preparations and on rat skin, and by comparing activity ratios the indices of discrimination were determined (Gaddum, 1955). The results of such comparisons showed that LPF differs from bradykinin, kallikrein, substance P or the globulin permeability factors prepared from rat and guinea-pig serum. Comparisons of the activity of LPF and these other permeability factors on rat skin in the presence of specific antagonists confirmed these results.

LPF was found to be stable at 4° for long periods, and to withstand heating at 56° for 30 minutes. The present evidence suggests that LPF is a large molecular substance since it fails to pass through dialysis tubing. However, it must be stressed that it may be a small molecule bound to protein and thus exhibit the properties of a larger molecule. Further work on the chemical purification of this crude extract will be required before it is possible to determine whether LPF is a permeability factor in its own right or whether it is an enzyme capable of forming such a substance.

The permeability increasing activity was present in approximately the same amount in all guinea-pig lymph node cells irrespective of the location of the node or the immunologic state of the animal. Thus extracts of the same number of cells from normal animals gave a similar response in rat skin as cells from guinea-pigs sensitized to tuberculin for 1, 2, 3 or 4 weeks. However, following sensitization the cell content of a node draining the site of injection may be increased from 5–10 million cells to 150–250 million cells. Thus the total amount of this factor in the whole animal is markedly raised during sensitization.

It is of interest to note that the amounts of LPF which will produce a response in rat skin are derived from approximately the same number of cells as are required to elicit a passive transfer of tuberculin hypersensitivity in the guinea-pig by the method of Metaxas (Metaxas and Metaxas-Buehler, 1955), in which sensitized cells are injected together with antigen into the skin of a normal animal, and a typical inflammatory reaction is produced 24 hours later. In experiments of this kind it is usually found that the intradermal injection of cells alone gives a small inflammatory response which is proportional to the number of cells injected (Metaxas and Metaxas-Buehler, 1955; Baum, Boughton, Mongar and Schild, 1961; Boughton and Schild, 1962). In the light of present work this could be explained by slow leakage of the permeability factor from the cells as they die, compared with the much faster liberation from the sensitized cells in the presence of the appropriate antigen.

The finding that sensitized and non-sensitized cells contain equivalent amounts of LPF is of interest since it is not at all clear from experiments recently reported that sensitized cells are specifically attracted to sites of antigen injection. Labelled sensitized cells transferred systemically to a normal animal became lodged in the lymphoid tissues of the recipients but were not noticeably increased in number compared to host cells at skin test sites (Turk, 1962; Hamilton and Chase, 1962). However Najarian and Feldman (1961) claimed that the labelled donor cells did accumulate at the site of antigen injection. It is possible that the 'sensitization' may be transferred in the recipient from donor to host cell in a similar manner to that indicated by the *in vitro* experiments of Mannick and Egdahl

(1962) in which extracts of cells capable of accelerating rejection of homograft were shown to enable normal cells to carry out this reaction.

In postulating a role for LPF in the delayed reaction and its associated inflammation the consistently potent activity of LPF on the skin of animals such as rats and mice which do not readily exhibit delayed reactions and the much more variable activity on guinea-pig skin presents a problem. It appears that the difference in these animals may lie partly in the immunological response to the initial sensitization since Flax and Waksman (1962) have shown that typical delayed reactions can be obtained in rats provided the sensitization is efficient and an adequate concentration of antigen is used for challenge, and Crowle (1960) has demonstrated delayed reactions to tuberculin in mice which could be passively transferred with lymph node cells. A further point to be considered is the presence of inhibitors or inactivators such as have been described for other large biologically active substances and which may vary according to species. Thus we have found that incubation with fresh guinea-pig plasma markedly reduced the effect of LPF on rat permeability, indicating the presence in guinea-pig serum of a substance which rapidly antagonizes or inactivates LPF.

The role of LPF in the delayed hypersensitivity reaction can perhaps be pictured as follows. Injection of antigen into the skin of sensitized animals leads to accumulation of mononuclear cells which contain LPF. It is possible that the function of the antigen is simply to attract the cells and that the release of LPF is a consequence of the ensuing stasis. On the other hand the antigen may produce more profound biochemical and cytological changes which cause the cells to release their LPF content. In either case, the build-up of a sufficiently high concentration of LPF would be manifest as a delayed inflammatory reaction. It must be stressed, however, that although the presence of a vascular permeability increasing agent in cells which are thought to play some part in the inflammatory response is a suggestive finding, there is at present no direct evidence that LPF participates in these reactions.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council and the Helen Hay Whitney Foundation.

REFERENCES

BAUM, J., BOUGHTON, B., MONGAR, J. L. and SCHILD, H. O. (1961). 'Autosensitization by sperm in guinea pigs.' *Immunology*, **4**, 93.

BOUGHTON, B. and SCHILD, H. O. (1962). 'Transfer of delayed hypersensitivity by lymph node cells in testis autosensitization.' *Immunology*, **5**, 522.

BOURA, A., MONGAR, J. L. and SCHILD, H. O. (1954). 'Improved automatic apparatus for pharmacological assays on isolated preparations.' *Brit. J. Pharmacol.*, **9**, 24.

CROWLE, A. J. (1960). 'Tuberculin skin reactions in mice hypersensitized by vaccination with living avirulent tubercle bacilli.' *Amer. Rev. resp. Dis.*, **81**, 893.

FISHER, J. P. and COOKE, R. A. (1958). 'Experimental and allergic contact dermatitis.' *J. Allergy*, **29**, 396.

FLAX, M. H. and WAKSMAN, B. H. (1962). 'Delayed cutaneous reactions in the rat.' *J. Immunol.*, **89**, 496.

GADDUM, J. H. (1955). *Polypeptides which Stimulate Plain Muscle*. Livingstone, Edinburgh.

HAMILTON, L. D. and CHASE, M. W. (1962). 'Labelled cells in the cellular transfer of delayed hypersensitivity.' *Fed. Proc.*, **21**(2), 40.

HOLDSTOCK, P. J., MATHIAS, A. P. and SCHACHTER, M. (1957). 'A comparative study of kinin, kallidin and bradykinin.' *Brit. J. Pharmacol.*, **12**, 149.

INDERBITZIN, T. (1961). 'The mechanisms involved in histamine release and histamine increase in allergic skin reactions.' *Int. Arch. Allergy*, **18**, 85.

JOHANOVSKY, J. (1959). 'Formation of pyrogenic substances in incubation of hypersensitive rabbit cells with tuberculin *in vitro*.' *Folia microbiol.*, **4**, 286.

JOHANOVSKY, J. (1961). 'Production of pyrogenic substances by extracts of delayed hypersensitive cells mixed with antigen *in vitro*.' *Nature (Lond.)*, **160**, 355.

JOHANOVSKY, J. and SKVARIL, F. (1962). 'Inhibition of the formation of hypersensitivity pyrogen from delayed hypersensitive cell extract by protease inhibitors *in vitro*.' *Immunology*, **5**, 469.

JUDAH, J. D. and WILLOUGHBY, D. A. (1962). 'A

quantitative method for the study of capillary permeability: extraction and determination of trypan blue in tissues.' *J. Path. Bact.*, **83**, 567.

MANNICK, J. A. and EGDAHL, R. H. (1962). 'Ribonucleic acid in "transformation" of lymphoid cells.' *Science*, **137**, 976.

METAXAS, M. N. and METAXAS-BUEHLER, M. (1955). 'Studies on the cellular transfer of tuberculin sensitivity in guinea pigs.' *J. Immunol.*, **75**, 333.

MONGAR, J. L. and SCHILD, H. O. (1957). 'Inhibition of the anaphylactic reaction.' *J. Physiol.*, **135**, 301.

NAJARIAN, J. S. and FELDMAN, J. D. (1961). 'Passive transfer of tuberculin sensitivity by tritiated thymidine-labelled lymphoid cells.' *J. exp. Med.*, **114**, 779.

SCHAYER, R. W. (1959). 'Histamine metabolism in the mammalian organism.' *Mechanisms of Hypersensitivity*, p. 227. Little-Brown, Boston.

SCHAYER, R. W. and GANLEY, O. H. (1959). 'Adaptive increase in mammalian histidine decarboxylase activity in response to non-specific stress.' *Amer. J. Physiol.*, **197**, 721.

SPECTOR, W. G. (1956). 'The mediation of altered capillary permeability in acute inflammation.' *J. Path. Bact.*, **72**, 367.

TURK, J. L. (1962). 'The passive transfer of delayed hypersensitivity in guinea pigs by transfusion of isotopically-labelled lymphoid cells.' *Immunology*, **5**, 478.

WILHELM, D. L., MILES, A. A. and MACKAY, M. E. (1955). 'Enzyme like globulins from serum reproducing the vascular phenomena of inflammation. II. Isolation and properties of the permeability factor and its inhibitor.' *Brit. J. exp. Path.*, **36**, 82.

WILLOUGHBY, D. A., BOUGHTON, B., SPECTOR, W. G. and SCHILD, H. O. (1962). 'A vascular permeability factor extracted from normal and sensitized guinea pig lymph node cells.' *Life Sciences*, (1962), No. 7, 347.