J. Pharm. Pharmac., 1975, **27**, 106–112 *Received September 5*, 1974

Effects of a human plasma fraction on leucocyte migration into inflammatory exudates

A. W. FORD-HUTCHINSON, M. J. H. SMITH, P. N. C. ELLIOTT, J. P. BOLAM, J. R. WALKER, A. A. LOBO, J. K. BADCOCK, A. J. COLLEDGE AND F. J. BILLIMORIA

Department of Biochemical Pharmacology, King's College Hospital Medical School, Denmark Hill, London SE5 8RX, U.K.

A fraction prepared from normal human plasma inhibits the migration of polymorphonuclear and mononuclear leucocytes into inflammatory exudates produced by the intrapleural injection of carrageenan or turpentine and by the subcutaneous implantation of polyvinyl sponges in the rat. The mechanism of the effect does not involve complement depletion.

A fraction prepared from normal human plasma shows anti-inflammatory activity in a number of acute paw oedema tests and in the adjuvant arthritis reaction in the rat (Elliott, Bolam & others, 1974). The mechanism of the anti-inflammatory action in carrageenan-induced inflammatory reactions does not involve specific interactions with either histamine, 5-hydroxytryptamine (5-HT), kinins or the prostaglandin system (Bolam, Elliott & others, 1974; Smith, Ford-Hutchinson & others, 1974). The fraction was active in the reversed passive Arthus reaction in the rat and rabbit but not against passive cutaneous anaphylaxis (Bolam, Ford-Hutchinson & others, 1974). Since the Arthus reaction, unlike passive cutaneous anaphylaxis, is considered to be a model of neutrophil and complement-mediated injury (Cochrane & Janoff, 1974) the effects of the plasma fraction on leucocyte migration into several types of inflammatory exudate and on complement have been investigated in the present work.

MATERIALS AND METHODS

Animals. Female albino Wistar rats (Oxfordshire Laboratory Animal Colonies Ltd.), 150–200 g were used for anti-inflammatory testing and for the production of inflammatory exudates. Guinea-pigs of the King's College Hospital strain were used as the source of fresh serum for the *in vitro* complement studies.

Plasma fraction. The procedure was that described by Ford-Hutchinson, Insley & others (1973). The plasma fraction used in the present work was their fraction II. Each preparation was tested for anti-inflammatory activity in groups of 5 rats by the carrageenan-induced paw oedema test and only those in which the intravenous administration of 1 ml per rat caused a minimum reduction of 50% in the mean paw swelling at 3 h were used. All injections of either the plasma fraction or of 0·9% (w/v) NaCl solution (saline) were filtered through Millipore Millex filter units, type GS 0·22 μm pore size, before being given intravenously into a tail vein.

Pleural exudates. The techniques used for the production, collection and the counting of polymorphonuclear and mononuclear leucocytes in the pleural exudates were based on those described by Hurley, Ryan & Friedman (1966) and Di Rosa, Giroud & Willoughby (1971). Each rat received an intrapleural injection either of 0·1 ml of Oil of Turpentine (BDH) or of 0·15 ml 1% (w/v) carrageenan (Viscarin Marine Colloids), immediately preceded by an intravenous injection of 1 ml either of plasma fraction or of saline and was either killed after 5 h or received a second injection of plasma fraction or saline at 8 h and was killed at 24 h.

Sponge implantation. Two or four preweighed polyvinyl sponges (1·2 × 1·2 × 0·5 cm, wt 15 ± 1 mg; foam sheet, Woolworth) were implanted according to the directions of Saxena (1960). Each rat received an intravenous injection of 1 ml, either of plasma fraction or of saline immediately before the sponges were inserted and was killed at either 2·5, 5·0 or 7·5 h, or received a second injection of plasma fraction or saline and was killed at 18 h, or received five injections of plasma fraction or saline at 8, 24, 32, 48 and 56 h and was killed at 72 h. Further experiments were performed on rats killed at 5 h in which each rat, fasted overnight, received either acetylsalicylic acid (Sigma) 150 mg kg^{-1}, phenylbutazone (Geigy) 50 mg kg^{-1}, or indomethacin (Merck, Sharp & Dohme) 3 mg kg^{-1}, orally 1 h before the sponges were implanted. All drugs were administered in either 1 or 2 ml of saline, the corresponding control animals being given an equivalent quantity of the vehicle.

The protein content of the fluid which had accumulated in the implanted sponges was estimated by the Biuret reaction, the absorption at 550 nm being measured in a Unicam SP800 spectrophotometer. Each sponge was carefully removed to avoid bleeding, placed in a 5 ml plastic syringe, the fluid squeezed gently into a volumetric flask and the process repeated, after the successive addition of 0·5 ml quantities of saline into the syringe, to make the final volume up to 5·0 ml. After centrifugation at 1500 g for 10 min, aliquots of the supernatant were removed for analysis of the protein. Total differential counting of leucocytes in the sponge fluid were performed as for the pleural exudates except that the sponges were first incubated for 30 min at 37° with 0·5 ml of 0·5% (w/v) trypsin (Sigma Type III) in Hank's medium at pH 7·4. The amount of granuloma formation on the sponges implanted for 72 h was measured by the difference in weight between the sponge, dried to constant weight at 80°, before and after implantation. A further test of granuloma formation was studied using the cotton-wool pellet method of Winter, Risley & Nuss (1963).

Complement measurement. The circulating titre of total haemolytic complement ($C'H_{50}$) was measured by the method of Mayer (1961) in samples of serum obtained from groups of normal rats, from animals with implanted sponges, and from rats with carrageenan-induced paw oedema. Each animal had received an intravenous injection of 1 ml of either saline or plasma fraction, the groups being killed at various time intervals. The effects of the *in vitro* addition of varying quantities of the plasma fraction on the activation of the haemolytic complement system by an antigen-antibody reaction (prepared from bovine serum albumin and rabbit anti-bovine serum albumin antiserum, Hoechst Pharmaceuticals), by heat aggregated γ-globulin (prepared from Cohn fraction II, Sigma) and by zymosan (Sigma) was investigated by the methods described by Gewurz, Shin & Mergenhagen (1968). Total haemolytic complement was also measured in the undiluted exudate in some of the implanted sponge experiments.

RESULTS

Pleural exudates

The results for the carrageenan-induced pleurisy, given in Table 1, show that the intravenous injection of the plasma fraction caused a significant decrease in the numbers of both polymorphonuclear and mononuclear leucocytes in the pleural exudates. Polymorphs predominated in the 5 h exudate and mononuclears in the 24 h exudate. Similar patterns of cell response and suppression of the migration by the plasma fraction were observed in the turpentine-induced pleural exudates.

Table 1. *Effects of plasma fraction on leucocyte counts in carrageenan-induced pleural exudate.* Results given as means and s.d. and expressed as counts $\times 10^3$ per mm^3 of pleural exudate. In this and subsequent tables results marked * show a statistically significant difference between the plasma fraction and corresponding control (saline-injected) animals. Each group contained 10 rats.

	Number of cells			
	5 h exudate		24 h exudate	
Group	Polymorphs	Mononuclears	Polymorphs	Mononuclears
Control	17·31 s.d. 1·23	4·72 s.d. 2·82	6·37 s.d. 2·89	9·98 s.d. 3·10
Plasma fraction	5·20 2·04*	1·43 0·84*	3·27 1·51*	4·06 0·40*

* $P < 0.01$.

Sponge exudates

Administration of the plasma fraction significantly inhibited both the accumulation of protein and the cellular infiltration into the sponge exudate. The protein content of the exudate was reduced by about 50% over the period 2·5 to 7·5 h but was not affected at the later time intervals (Table 2). The effect on leucocyte migration was greater and more prolonged (Table 3). The inhibitory effects on the migration of polymorphonuclear and mononuclear leucocytes were about 90% over the period 2·5 to 18 h and about 60% at 72 h irrespective of which cell type predominated in the exudate.

In contrast to the almost complete shutdown of cellular emigration caused by the plasma fraction in the 5 h exudate the administration of some conventional anti-inflammatory drugs had much less effect (Table 4).

Table 2. *Effects of plasma fraction on protein content of sponge exudate.* Results given as means and s.d. and expressed as mg of total protein in sponge exudate. Each group contained 5 rats.

Time (h)	Control	Plasma fraction
2·5	6·0 s.d. 1·3	4·1 s.d. 0·9*
5·0	7·0 2·3	3·8 1·1*
7·5	9·1 1·6	6·5 1·5*
18·0	15·9 1·1	15·2 1·5
72·0	13·4 2·6	12·6 1·0

* $P < 0.05$.

Table 3. *Effect of plasma fraction on leucocyte migration into sponge exudate.* Results given as means and s.d. and expressed as counts $\times 10^4$ per ml of sponge exudate after incubation with trypsin (see text). Each group contained 5 rats.

	Number of cells			
	Polymorphs		Mononuclears	
Time (h)	Control	Plasma fraction	Control	Plasma fraction
2·5	125 s.d. 55	6 s.d. 7*	13 s.d. 8	1 s.d. 1*
5·0	170 98	9 8*	31 15	2 1*
7·5	501 171	38 17*	185 98	12 8*
18·0	1379 323	184 64*	724 278	29 11*
72·0	254 117	79 29*	799 131	305 54*

* $P < 0{\cdot}01$.

Table 4. *Effects of anti-inflammatory drugs on the protein content and leucocyte migration into the 5 h sponge exudate.* Results given and expressed as in Tables 2 and 3, each group contained 10 rats.

		Number of cells	
Treatment	Protein content	Polymorphs	Mononuclears
Control	6·5 s.d. 1·4	184 s.d. 60	47 s.d. 19
Aspirin	3·8 0·9*	108 32*	35 14
Phenylbutazone	5·4 2·0	115 33*	31 7
Indomethacin	5·9 1·4	106 35*	30 20

* $P < 0{\cdot}01$.

Three of these drugs, aspirin, phenylbutazone and indomethacin were given orally in doses equivalent to those known to cause significant inhibitory effects in a variety of other experimental anti-inflammatory reactions (Domenjoz, 1971). Aspirin was the only one to reduce the protein content of the sponge exudate but all three significantly reduced the migration of polymorphonuclear leucocytes by about 40%. This finding does not agree with the conclusion of Di Rosa, Sorrentino & Parente (1972) that conventional antirheumatic drugs affect mononuclear but not polymorphonuclear leucocyte emigration into inflammatory exudates.

Repeated injections of the plasma fraction did not significantly affect the formation of granuloma tissue either in the 72 h sponge system (control: dry wt of granuloma 118 s.d. 36 mg; plasma fraction 97 s.d. 29 mg n = 10) or in the cotton-wool pellet test (control: 53 s.d. 12 mg; plasma fraction 52 s.d. 14 mg n = 40). In the latter reaction the daily administration of 1·5 mg of indomethacin by intraperitoneal injection caused a significant decrease in the granuloma formation (control: 53 s.d. 12 mg; indomethacin 41 s.d. 11 n = 30 $P < 0{\cdot}05$).

Complement measurements

The intravenous administration of 1 ml of plasma fraction instead of saline had no effect on the circulating titre of total haemolytic complement, when measured at time intervals ranging from 0–6 h, in groups of normal rats in animals with implanted

sponges, and during the development of carrageenan-induced paw swelling. Although the $C'H_{50}$ values in the serum were not affected, the local complement levels in the implanted sponge exudates at 5 h were significantly increased by intravenous injection of the plasma factor ($C'H_{50}$ serum: saline control 72 s.d. 15, plasma factor 68 s.d. 8 $n = 20$; $C'H_{50}$ sponge exudate control 8·7 s.d. 2·2, plasma factor 12·7 s.d. 3·8 $n = 20$ $P < 0{\cdot}01$).

Activation *in vitro* of either the classical or alternate pathways of complement in fresh guinea-pig serum by either antigen-antibody, heat aggregate γ-globulin or zymosan, assessed by measuring the residual total haemolytic complement titre (see Gewurz & others, 1968), was not affected by the previous addition of amounts of the plasma fraction ranging from 0·1 to 1·0 ml.

DISCUSSION

The results of the present work show that the anti-inflammatory fraction prepared from normal human plasma causes a substantial inhibition of leucocyte migration into inflammatory exudates. A significant effect is seen in the carrageenan-induced pleural exudate (Table 1) but is even more evident, the inhibition amounting to about 90%, in the experiments with implanted non-resorbable sponges (Table 3). The inflammatory reaction in the sponge model, as assessed by the accumulation of proteins and cellular infiltration into the sponge exudate, is probably an index of local mechanical injury due to implantation (Saxena, 1960) but, unlike the various types of pleurisy, it does not involve the use of locally toxic materials and serves as a relatively mild inflammatory stimulus.

The inhibitory action of the intravenous injection of the plasma fraction on leucocyte migration into the sponge exudate affects both polymorphonuclear and mononuclear leucocytes. There is general agreement that both types of leucocytes escape from blood vessels into the inflammatory exudate and that an infiltrate initially composed of polymorphs is frequently transformed to one consisting mainly of mononuclear cells. However, the available evidence does not prove if the emigration of the two cell types are either related or independent processes and if they are influenced by either the same or separate mediators (Hurley & others, 1966). An important group of such mediators thought to induce directed migration of leucocytes through the tissue extracellular space, i.e. into exudates, are the various chemotactic factors derived from the complement system (Ward, 1970). Although chemotaxis is an extremely difficult phenomenon to prove under direct observation *in vivo* the circumstantial evidence for it is overwhelming (Grant, 1973). One possible mechanism of the effect of the plasma fraction on leucocyte migration could be that it depletes circulating complement thus preventing the sustained release of chemotactic fragments by the inflammatory stimulus. Intravenous administration of the plasma fraction either to normal rats or to animals exposed to inflammatory reactions, carrageenan-induced paw oedema and sponge implantation, showed no evidence of depletion of the circulating titre of total haemolytic complement. It has been shown by Wiener, Lendvai & others (1973) that for an inhibition of leucocyte migration into implanted sponge exudates in the rat to occur, a 97% depletion of serum complement levels by treatment with purified cobra venom factor is required. The plasma fraction therefore does not act by depleting serum complement levels *in vivo*.

The *in vitro* complement experiments showed that the fraction does not appear to protect the complement system in guinea-pig serum from being activated by either immunological or non-immunological stimuli. These experiments were performed since the complement levels in the sponge exudates from the rats injected with plasma fraction were higher than the corresponding control values. This result could not be explained on the basis of more accumulation of proteins, including complement, into the sponge exudates in the plasma fraction-treated rats since the reverse occurred (Table 2) and suggested that there may have been inhibition of activation of local complement levels in the exudates.

Although administration of the plasma fraction does not deplete total serum haemolytic complement levels *in vivo* it may exert a selective action on the release of specific chemotactic fragments, e.g. $C3_a$ and $C5_a$, after activation of the complement cascade. Alternatively it may block the effects of these factors on receptor systems on the leucocyte cell surfaces. The inhibitory actions of the plasma fraction on leucocyte migration into the inflammatory exudates may also result from an interaction at earlier stages such as the movement of the leucocytes through the membranes of small vascular beds and the adhesion (pavementing) of white cells to the capillary endothelium.

It is also of interest that the plasma factor inhibits the accumulation of protein as well as leucocytes into the sponge exudates. This finding suggests that at least one of its effects is intravascular, i.e. on the increased vascular permeability to circulating proteins, a similar action being observed in other types of inflammatory reactions (Bolam & others, 1974). Although there is evidence (Hurley, 1972) that leucocyte emigration and increased vascular permeability are completely separable phenomena, it is possible that the plasma fraction suppresses both aspects of inflammation by the same mechanism.

Administration of the plasma fraction did not affect granuloma formation either on sponges present for 72 h, although an inhibitory action on polymorphs and mononuclear counts in the exudate was still evident (Table 3), or on implanted cotton-wool pellets. This finding suggests that there was no interference with either the proliferation (Swingle & Shideman, 1972) or the persistence of the emigrated cells in these types of chronic granulomatous inflammation (see Spector, 1974; Willoughby & Di Rosa, 1971).

Acknowledgements

We wish to thank the National Research Development Corporation, King's College Hospital Research Committee, the Wates Foundation, the Nuffield Foundation and Abbott Laboratories Ltd. for financial support, the Blood Transfusion Centre, Tooting, London, S.W.17 for the plasma and Dr. A. K. Clarke for helpful advice.

REFERENCES

BOLAM, J. P., ELLIOTT, P. N. C., FORD-HUTCHINSON, A. W. & SMITH, M. J. H. (1974). *J. Pharm. Pharmac.*, **26**, 434–440.

BOLAM, J. P., FORD-HUTCHINSON, A. W., ELLIOTT, P. N. C. & SMITH, M. J. H. (1974). *Ibid.*, **26**, 660–661.

COCHRANE, C. G. & JANOFF, A. (1974). In: *The Inflammatory Process, III*, pp. 86–155. London: Academic Press.

DI ROSA, M., GIROUD, J. P. & WILLOUGHBY, D. A. (1971). *J. Path.*, **104**, 15–29.

DI ROSA, M., SORRENTINO, L. & PARENTE, L. (1972). *J. Pharm. Pharmac.*, **24**, 575–576.

DOMENJOZ, R. (1971). In: *Rheumatoid Arthritis*, pp. 513–550. London: Academic Press.

ELLIOTT, P. N. C., BOLAM, J. P., FORD-HUTCHINSON, A. W. & SMITH, M. J. H. (1974). *J. Pharm. Pharmac.*, **26**, 751–752.

FORD-HUTCHINSON, A. W., INSLEY, M. Y., ELLIOTT, P. N. C., STURGESS, E. A. & SMITH, M. J. H. (1973). *Ibid.*, **25**, 881–886.

GEWURZ, H., SHIN, H. S. & MERGENHAGEN, S. E. (1968). *J. exp. Med.*, **128**, 1049–1057.

GRANT, L. (1973). In: *The Inflammatory Process*, **2**, pp. 205–244. London: Academic Press.

HURLEY, J. V. (1972). *Acute Inflammation.* London: Churchill Livingstone.

HURLEY, J. V., RYAN, G. B. & FRIEDMAN, A. (1966). *J. Path.*, **91**, 575–587.

MAYER, M. M. (1961). In: *Experimental Immunochemistry*, pp. 133–240. Springfield, Illinois: Charles C. Thomas.

SAXENA, P. N. (1960). *Archs int. Pharmacodyn. Thér.*, **126**, 228–237.

SMITH, M. J. H., FORD-HUTCHINSON, A. W., ELLIOTT, P. N. C. & BOLAM, J. P. (1974). *J. Pharm. Pharmac.*, **26**, 692–698.

SPECTOR, W. G. (1974). In: *The Inflammatory Process*, **3**, 277–289. London: Academic Press.

SWINGLE, K. F. & SHIDEMAN, F. E. (1972). *J. Pharmac. exp. Ther.*, **183**, 226–234.

WARD, P. A. (1970). *Arthritis Rheum.*, **13**, 181–186.

WIENER, S., LENDVAI, S., ROGERS, B., URIVETSKY, M. & MEILMAN, E. (1973). *Am. J. Path.*, **73**, 807–816.

WILLOUGHBY, D. A. & DI ROSA, M. (1971). In: *Immunopathology of Inflammation*, pp. 28–38. Amsterdam: Excerpta Medica.

WINTER, C. A., RISLEY, E. A. & NUSS, G. W. (1963). *J. Pharmac. exp. Ther.*, **141**, 369–376.