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Effects of a human plasma fraction on the release of chemotactic factors and anaphylatoxin from complement

J. R. WALKER, J. K. BADCOCK, A. W. FORD-HUTCHINSON, M. J. H. SMITH 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 release of chemotactic factors and anaphylatoxin when complement is activated by the alternate but not by the classical pathway. The fraction gave no interference with the actions of the released substances on either leucocytes or guinea-pig isolated ileum. The possible relation of this effect to the mechanism of the anti-inflammatory actions of the plasma fraction is discussed.

A fraction prepared from normal human plasma (Ford-Hutchinson, Insley & others, 1973) shows anti-inflammatory activity in a number of animal models (Elliott, Bolam & others, 1974). Its activity in the carrageenan-induced paw oedema reaction in the rat does not involve an interference with either the release or action of chemical mediators of inflammation such as histamine, 5-hydroxytryptamine, kinins or prostaglandins (Bolam, Elliott & others, 1974; Smith, Ford-Hutchinson & others, 1974). The fraction is not active in inflammatory responses in which these mediators play the more prominent role but shows marked and reproducible activity in reactions in which the emigration of circulating leucocytes into inflammatory exudates is a major factor (Ford-Hutchinson, Smith & others, 1975). An important group of substances thought to induce the directed migration of leucocytes from the blood into inflammatory exudates are the chemotactic peptides derived from the complement system (Ward, 1974). The active substance in the plasma fraction could interfere with chemotaxis by one or more of several mechanisms. The results of previous work (Ford-Hutchinson & others, 1975) have shown that the fraction neither depletes complement levels *in vivo* nor prevents the activation of the complement system *in vitro* when this is assessed by measuring the residual total haemolytic titre. In the present work we have studied its effects on the selective release of chemotactic factors and anaphylatoxin in fresh rat, guinea-pig and human serum using an antigen-antibody complex as the activator of the classical pathway of complement and zymosan and endotoxin as activators of the alternate pathway (see Vogt, 1974). The effects of the plasma fraction on the response *in vitro* of human and rat leucocytes to chemotaxis and on other aspects of their behaviour and function were also investigated. Preliminary accounts of parts of this work have already been published (Badcock, Ford-Hutchinson & others, 1975; Walker, Smith & others, 1975).

MATERIALS AND METHODS

Preparation of active fraction. Pooled citrated human plasma, obtained from the Blood Transfusion Centre, Tooting, London S.W.17, was centrifuged at 1500 *g* for 45 min at 4° in an MSE Mistral 4L centrifuge. The supernatant was ultrafiltered at 20° in an Amicon DC2 hollow fibre cell fitted with a H1DP 10 cartridge with a molecular weight cut-off of 10 000 daltons. Portions (1 litre) of ultrafiltrate were reduced to 150 ml at 50° under reduced pressure, the concentrated ultrafiltrate applied to a 35 × 8·5 cm column (Wright Scientific Ltd., Kenley, Surrey) containing Sephadex G25 fine, and eluted with distilled water at 20°. The eluate (15 ml min^{-1} flow rate) was monitored at 254 nm using a Uvicord I (LKB Instruments Ltd., Croydon). Portions (50 ml) were collected and combined into fractions as described previously (Ford-Hutchinson & others, 1973). Fraction II from four separate column runs was combined, reduced to 50 ml at 50° under reduced pressure and applied to a 2·2 × 2·2 cm column of Sephadex A25 ion exchange resin. The column was eluted at 0° with 12 ml of distilled water followed by 120 ml of a mixture containing 5% (v/v) acetic acid and 0·45% (w/v) NaCl (flow rate 1 ml min^{-1}), the eluate being monitored at 254 nm using a Uvicord II. Portions (12 ml) were collected at 2° and the final peak showing absorption at 254 nm was collected, bulked and its pH quickly adjusted to pH 7·0 by the addition of 4 M NaOH. The volume was reduced to 10 ml at 50° under reduced pressure and the concentrate applied to a 2·2 × 75 cm column of Sephadex G25 fine, eluted at 0° with distilled water (flow rate 1 ml min^{-1}) and the portion corresponding to the fraction II of Ford-Hutchinson & others (1973) was collected and adjusted to a final volume of 100 ml. This preparation was used throughout the present work. It was stored in liquid nitrogen and routinely assayed by the sponge implantation technique described by Ford-Hutchinson & others (1975) using one sponge implanted for 5 h per rat and an injection volume of 1 ml of the plasma fraction preparation. Inactivated plasma fraction was prepared by heating 5 ml of plasma fraction with 1 ml of 9 M NH_4OH solution for 1 h at 100°, evaporating to dryness under reduced pressure and dissolving the residue in 0·9% (w/v) NaCl solution.

Measurement of chemotaxis. Blood obtained either by cardiac puncture from male albino Wistar rats (Oxfordshire Laboratory Animal Colonies Ltd.) 250–400 g, or by venepuncture from normal healthy subjects was allowed to clot and the serum separated. Further blood samples from both sources were collected into heparinized containers, an equal volume of Plasmagel (Laboratoire Roger Bellon, Neuilly, France) added, the mixture allowed to sediment for 10 min at 37°, the leucocyte-rich plasmagel layer aspirated, centrifuged at 300 *g* for 6 min, the supernatant discarded and the cellular sediment suspended in 0·2% (w/v) NaCl solution for 20 s to haemolyse any red cells present (Broder, Tackaberry & others, 1974). After the addition of an equal volume of 1·6% (w/v) NaCl solution, the suspension was centrifuged at 300 *g* for 6 min, the supernatant removed and the leucocytes resuspended in Medium 199 (Wellcome Reagents Ltd., Beckenham). The final leucocyte suspension contained 3 to 5 × 10^6 cells ml^{-1} and was kept on ice until used. Polystyrene disposable chemotactic chambers (Adaps Inc., Dedham, Mass., U.S.A.) and Millipore filters with 3 μm pore size (Millipore Corp., Bedford, Mass., U.S.A.) were used. Rat or human leucocyte suspension (1 ml) was placed in the upper chamber and 0·9 ml of the corresponding serum in the lower chamber. To the serum was added either

0·1 ml of 0·9% (w/v) NaCl solution (saline control) or 0·1 ml of saline containing one of the following activators: Zymosan (50 μg, Sigma Chemical Co.), *E. coli* 011B4 lipopolysaccharide endotoxin (50 μg, Difco Laboratories, Detroit, U.S.A.), or antigen-antibody complex (50 μg of human serum albumin: anti-human serum albumin, Sigma Chemical Co. fraction V: Hoechst Pharmaceuticals, U.K.) prepared by the method of Gewurz, Shin & Mergenhagen (1968). The concentrations of activators were chosen as a result of preliminary experiments using a range of Zymosan, endotoxin and antigen-antibody concentrations. The mixtures of serum and activator in the lower chambers were incubated for 1 h at 37° to ensure release of the chemotactic factors and the chambers were then assembled, incubated for a further 2 h at 37°, the filters removed and the cells which had penetrated the filter fixed, stained and counted according to Boyden (1962) and Goetzl & Austen (1972). Further experiments were made in which varying amounts of the plasma fraction preparations were added to serum either before or after release of the chemotactic factors, i.e. either before or after the initial incubation with the activator, or to the cell suspension in the upper chamber. Each preparation of plasma fraction was independently tested for their effects both on the accumulation of leucocytes in single sponges implanted for 5 h subdermally in the intact rat (Ford-Hutchinson & others, 1975) and on the depletion of the total haemolytic titre of either rat or human serum by Zymosan (50 μg ml^{-1}).

Assay of anaphylatoxin. Blood obtained either by exsanguination of guinea-pigs of the King's College Hospital strain or by venepuncture from normal healthy subjects was allowed to clot and the serum separated. Samples of the sera (2 ml) were added to 0·2 ml of either 0·9% (w/v) NaCl solution (saline control) or plasma fraction preparation, followed by the addition of the activator, either 0·4 ml of sodium diethylbarbiturate buffer (pH 7·4, 0·147 M) containing 5 mg ml^{-1} Zymosan or 0·1 ml of saline containing 0·5 mg ml^{-1} of antigen-antibody complex (see section on measurement of chemotaxis). The mixtures were incubated at 37° for 30 min and the released anaphylatoxin was partially purified by precipitation with ethanol according to Kleine, Poppe & Vogt (1970). Control experiments were performed in which either no activator was added or 0·2 ml of either saline or plasma fraction were added to the serum-activator mixture after incubation. The anaphylatoxin was assayed using portions of guinea-pig ileum suspended in oxygenated Tyrode solution at 35° in a 10 ml organ bath. The sensitivity of the isolated tissue was determined using standard doses of histamine. Four to eight samples (0·1 ml) of anaphylatoxin preparation could be tested on each ileum before tachyphylaxis occurred. Additional control experiments were also made in which 1 ml of plasma fraction was added to the organ bath before the addition of the anaphylatoxin. The results were calculated in terms of the contractions induced by given amounts of histamine and expressed as percentage inhibitions of the values obtained with the serum-activator mixtures. In the experiments with human serum a further correction was made to allow for the contraction caused by the presence of the serum itself. Each experiment was repeated on at least four occasions.

Effects of plasma fraction on leucocyte suspensions. The effect of plasma fraction on the random motility of rat and human leucocytes was studied in the chemotactic chambers as described in the section on the measurement of chemotaxis except that

the incubation of the serum with activator stage was omitted and either saline (0·1 ml) or plasma fraction (0·1 ml) was added to both chambers. The cells which had penetrated the millipore filter were counted according to Van Epps, Palmer & Williams (1974). Further experiments involving random mobility were made by the capillary tube method (Maini, Roffe & others, 1973) and the abilities of the leucocyte preparation, either in the absence or the presence of varying amounts of the plasma fraction, to stain with trypan blue (Stevenson, 1973), to phagocytize Zymosan and ink granules (Böyum, 1968), to adhere to glass or to form rosettes with sensitized sheep red cells (Durance, Micheli & Fallet, 1974) were also investigated.

RESULTS

Effects of plasma fraction on the chemotaxis of leucocytes in vivo *and* in vitro

The preparations of plasma fraction used cause a significant inhibition ($P<0·001$) of the migration of leucocytes into the exudates found in inert porous sponges implanted subdermally for 5 h in the intact rat [control group (1 ml saline) no. of cells 29·1 s.d. $5·9 \times 10^4$ ml^{-1} of 5 h sponge exudate, n = 15; plasma fraction (1 ml) no. of cells 13·0 s.d. $1·7 \times 10^4$ ml^{-1}, n = 15]. When the plasma fraction was treated by boiling with ammonium hydroxide solution (inactivated plasma fraction) its ability to interfere with chemotaxis *in vivo* was lost (no. of cells 29·5 s.d. 3·4, n = 15). None of the plasma fractions affected the residual total haemolytic complement titre of fresh rat, human or guinea-pig serum after activation by Zymosan.

The results of the experiments with the chemotactic chambers are given in Table 1.

Table 1. *Effects of plasma fraction on the release of chemotactic factors from rat and human serum.* Results given as mean with s.d. and expressed as number of cells per high power field, ten fields were counted in each experiment and the number of experiments are given in brackets. The leucocyte suspensions (1·0 ml) were placed in the upper chamber and the corresponding serum (0·9 ml) after incubation with the appropriate activator, in the lower chamber. Additions of the plasma fraction (PF) or saline are indicated in the Table.

		Number of cells	
Activator	Addition to serum	Rat	Man
None	Saline (0·1 ml)	2·0 s.d. 0·4 (12)	1·9 s.d. 0·8 (9)
Zymosan	Saline (0·1 ml)	14·5 s.d. 3·4 (12)	23·5 s.d. 0·9 (15)
	PF (0·1 ml) before incubation	9·3 s.d. 2·5 (12)*	1·5 s.d. 1·2 (6)*
	PF (0·1 ml) after incubation	13·1 s.d. 2·9 (9)	24·4 s.d. 1·0 (6)
	Inactivated PF (0·1 ml) before incubation	14·7 s.d. 0·8 (9)	Not determined
	PF (0·1 ml) to cells in upper chamber	12·8 s.d. 4·1 (9)	26·8 s.d. 0·3 (6)
E. coli endotoxin	Saline (0·1 ml)	22·4 s.d. 0·7 (9)	Not determined
	PF (0·1 ml) before incubation	13·2 s.d. 0·7 (9)*	Not determined
Antigen-antibody	Saline (0·1 ml)	15·6 s.d. 1·0 (12)	22·2 s.d. 0·7 (12)
	PF (0·1 ml) before incubation	15·8 s.d. 0·7 (12)	22·0 s.d. 1·0 (12)

* Significant difference ($P < 0·01$) from the corresponding value with activator alone and in the Zymosan experiments from the values in which plasma fraction was either added after incubation of the chemotactic generating system, added to the cell suspension or when inactivated plasma fraction was used.

Each preparation of plasma fraction was studied on the same day as it was tested in the sponge experiments. When the plasma fraction was added to the serum and Zymosan mixture before incubation, i.e., before release of the complement-derived chemotactic factors, a significant decrease in the directed migration occurred in the experiments with either rat or human material. This effect was not observed when inactivated plasma fraction was used or when the plasma fraction was added either after incubation of the serum with the activator or to the cell suspensions in the upper chamber. Similar results were obtained with endotoxin as the activator in the rat system but not when the antigen-antibody complex was used to generate chemotactic factors from either rat or human serum. In the Zymosan experiments it appeared that the human serum-leucocyte system was more sensitive to the effects of the plasma fraction than the rat system and this was studied in more detail with serum from both species by adding varying amounts of the plasma fraction to the serum-Zymosan mixtures before incubation. The results (Table 2) show that there is at least a 20-fold difference in sensitivity between the experiments using human or rat material.

Table 2. *Effects of varying amounts of plasma fraction on the release of chemotactic factors from rat and human serum by Zymosan.* Results given and expressed as in Table 1.

Quantity (ml) of plasma fraction added to serum-zymosan mixture before incubation	Number of cells			
	Rat	Inhibition (%)	Man	Inhibition (%)
Control (none)	14·5 s.d. 3·4 (12)	0	23·5 s.d. 0·9 (15)	0
0·005	Not determined		15·2 s.d. 8·6 (9)*	40
0·01	13·3 s.d. 1·2 (9)	10	9·6 s.d. 6·9 (9)*	62
0·02	11·3 s.d. 0·7 (9)*	23	4·6 s.d. 1·9 (15)*	80
0·1	9·3 s.d. 2·5 (12)*	36	1·5 s.d. 1·2 (6)*	94

* Significant difference ($P < 0{\cdot}01$) from results of corresponding saline control.

Effects of plasma fraction on release of anaphylatoxin. The addition of 0·1 ml quantities of several preparations of the plasma fraction to 0·9 ml of either fresh guinea-pig or human serum before incubation with Zymosan caused a significant ($P < 0{\cdot}001$) reduction in the contractions induced in the isolated guinea-pig ileum preparation (% inhibition of contraction: guinea-pig serum 40 ± 8, $n = 5$; man 63 ± 1 $n = 4$). The contractions were not inhibited when the plasma fraction was added after the serum-activator incubation, when 1 ml of the plasma fraction was added to the organ bath or when antigen-antibody complex was used as the activator.

Effects of plasma fraction on leucocyte behaviour and function. The failure of the plasma fraction to significantly affect the directed migration of either the rat or human leucocytes when it was added to the cell suspensions in the upper chamber (Table 1) suggested that the plasma fraction does not act on the leucocyte cell surfaces to prevent chemotactic attraction. Further evidence that it has no effects on other aspects of leucocyte function and behaviour was provided by the results of the random migration experiments in the chemotactic chambers. When saline was added to both chambers the numbers of cells per high power field were: rat: $10{\cdot}0 \pm 0{\cdot}7$, $n = 12$,

man: 5·1 ± 0·6, n = 12 and when plasma fraction was added: rat: 9·8 ± 0·6, n = 12, man 5·2 ± 0·1, n = 12. In other experiments exposure of the leucocyte suspensions to quantities of plasma fraction ranging from 0·1 to 1·0 ml did not affect the percentage of cells staining with trypan blue, the number of ink and Zymosan granules phagocytized, or the numbers of leucocytes adhering to glass surfaces or forming rosettes.

DISCUSSION

The results of the present work show that in the rat the human plasma fraction inhibits the chemotaxis of leucocytes both *in vivo* and *in vitro*. The effective amounts of the plasma fraction in both types of experiment are comparable in that 1 ml was injected intravenously in each animal in the implanted sponge experiments, and may be considered to have been diluted by a factor of 10 in the circulation, whereas in the chemotactic chambers (Table 1) 0·1 ml was mixed with 0·9 ml of rat serum.

The site of action of the plasma fraction is on the selective release of chemotactic factors from complement components in the serum. The inhibitory effect on the directed migration of rat and human leucocytes in the chemotactic chambers occurred only when the fraction was added to the mixture of serum and complement activator before the incubation to release the chemotactic fragments. No inhibition was observed when the plasma fraction was added after the incubation nor when it was added to the leucocyte suspensions in the upper chambers of the apparatus (Table 1). Thus, when leucocytes were exposed to the plasma fraction *in vitro* their ability to respond to the released chemotactic factors was not impaired. The plasma fraction did not affect other aspects of leucocyte function and behaviour including random motility, phagocytosis and immune adherence. Furthermore, the plasma fraction interferes with the release of chemotactic fragments only when complement is activated by the alternate and not by the classical pathway (see Vogt, 1974). Thus the addition of the plasma fraction significantly inhibited the chemotaxis of rat or human leucocytes *in vitro* when either Zymosan or *E. coli* endotoxin were used as activators but not when the antigen-antibody complex was employed (Table 1). The results of the anaphylatoxin experiments support these conclusions. It has been suggested (see Nelson, 1974) that one of the peptides, C5a, released after the activation of complement possesses chemotactic activity and functions as an anaphylatoxin, i.e. it causes smooth muscle contraction with tachyphylaxis and histamine release. In the present work the plasma fraction was found to inhibit the release of anaphylatoxin from either guinea-pig or human serum when it was added before the incubation of the serum with Zymosan but not with the antigen-antibody complex.

The selective action of the plasma fraction on the release of peptide fragments, e.g. C5a, after activation of the complement cascade by the alternate pathway *in vitro* may be of some significance *in vivo*. Although chemotaxis is an extremely difficult phenomenon to prove by direct observation in the whole animal the circumstantial evidence for it appears to be overwhelming (Grant, 1973; Ramsey & Grant, 1974). When complement proteins and leucocytes have entered inflammatory exudates a vicious circle may be set up because the leucocytes contain not only enzymes capable of cleaving complement components, such as C5, to release chemotactic peptides but also contain a substance which has been shown to activate the complement system by the alternate pathway (Vogt, 1974; Goldstein & Weissmann, 1974). As soon as leucocytes have accumulated in the presence of extravascular complement they may

interact to both increase vascular permeability and generate more chemotactic factors thus amplifying and prolonging the inflammation. The persistence of an inflammatory response may be largely dependent on the secondary events mediated by the leucocytes which have accumulated at the site of the inflammatory insult. The anti-inflammatory activity of the plasma fraction in susceptible animal models, i.e. those associated with neutrophil emigration such as Arthus reactions and carrageenan-induced systems, could result from a selective interference with the release of complement-derived peptide fragments in this secondary phase. The results with the human serum-leucocyte system in the chemotactic chambers (Table 1) suggest that this may also apply to related conditions in man.

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