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Anti-inflammatory activity in human plasma

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Pooled human plasma contains a fraction, separable by ultrafiltration and column chromatography, which possesses anti-inflammatory activity in the carrageenan oedema test in the rat, may be partially purified by solvent extraction, has an apparent molecular weight below 1000, and is resistant to acid and proteolytic digestion.

A substance showing experimental anti-inflammatory activity has been isolated from human serum (McArthur, Smith & Freeman, 1972). The present paper describes a much simpler preparation of this material from pooled human plasma, its partial purification by extraction with acetone and ethanol, its ultrafiltration through several membranes, and its resistance to acidic, proteolytic and thermal degradation. A preliminary account of part of this work has been published (Elliott, Ford-Hutchinson & others, 1973).

MATERIALS AND METHODS

Preparation of active extract

Pooled citrated human plasma (1 litre), obtained from the Blood Transfusion Centre, Tooting, London, S.W.17, was centrifuged at 2000 *g* for 30 min at 4° in an MSE Mistral 4L centrifuge and the supernatant filtered without delay at 20° in an Amicon 2L cell under an atmosphere of N_2 at a pressure of 50 psi using a Diaflo PM 10 membrane until 200 ml of ultrafiltrate had collected. The volume of ultrafiltrate was reduced to 20 ml at 37° under reduced pressure, the concentrate applied to a 55 × 5 cm column containing Sephadex G 25 fine and eluted with distilled water at 20°. The eluate (2 ml min^{-1} flow rate) was monitored at 254 nm by means of a Uvicord I (LKB Instruments, Croydon), portions (20 ml) were collected and combined into fractions, designated I, II, III, IV and V, as described in the results. The eluate fractions were reduced to dryness at 37° under reduced pressure, dissolved in 6 ml of 0·9 % (w/v) NaCl solution and used for anti-inflammatory testing. Fractions II and IV were dissolved in water (10 ml) and re-run on the G 25 columns as for the original ultrafiltrate. Similar experiments were made in which either Diaflo UM 2 or UM 05 membranes were used instead of the PM 10 membranes.

Anti-inflammatory testing

The activity in the eluate fractions was assessed by the carrageenan paw oedema test in groups of five female rats (Winter, Risley & Nuss, 1962) (150–200 g) obtained from Oxfordshire Laboratory Animal Colonies (Southern) Ltd. In the control group each animal received 1 ml of 0·9 % (w/v) saline and in the experimental group each rat received 1 ml of the extract, prepared as described above. All the injections were filtered through Millipore Millex filter units, type GS 0·22 μm pore size, before

being given intravenously into a tail vein 30 min before the injection of 0·1 ml of 1·0% (w/v) carrageenan (Viscarin Marine Colloids) in 0·9% (w/v) NaCl in the plantar region of the right hind foot. Foot volumes were measured using a mercury plethysmograph (Arnold R. Horwell Ltd., London) immediately after the injection of the carrageenan (0 h) and at hourly intervals for 6 h. The results were calculated as mean percentage increases in the volume of the injected paw compared to the value at 0 h.

Stability of activity to heat, acid and proteolytic enzymes

The residue from fraction IV of the column eluate (see above) was heated in sealed tubes with either distilled water or 50% (v/v) HCl under an atmosphere of N_2 for 72 h at 110°. Most of the acid was removed under reduced pressure on a rotary evaporator and any remaining was neutralized by the addition of 2M NaOH solution before animal testing. Other samples of the residue from fraction IV were incubated with 25 mg of insoluble carboxymethyl cellulose-bound non-specific protease from *Streptomyces griseus* (Sigma Chemicals, Kingston-upon-Thames, Surrey, U.K.) at 40° for 24 h at pH 7·0 in an unbuffered solution. Enzyme activity was assayed at 61 units g^{-1} (1 unit will hydrolyse 1 μmol of *N*-(α)-benzoyl-L-arginine ethyl ester min^{-1} at pH 7·0 at 25°). After digestion the enzyme was removed by filtration on a sintered glass funnel. Similar experiments were performed using insoluble trypsin and papain.

Partial purification

Portions of the residue from fraction IV of the column eluate were extracted at 37° with six portions, each of 50 ml, of either acetone, ethanol or chloroform, the solvent being removed under reduced pressure. The acetone-extracted residue was also subsequently extracted with ethanol. Portions of the insoluble residues and extractable material were dissolved in 0·9% (w/v) NaCl and tested for anti-inflammatory activity by the carrageenan method. Other portions were examined by descending paper chromatography (Whatman No. 1 paper) and thin-layer chromatography (Polygram SILG/UV254 precoated sheets, Camlab Ltd., Cambridge) using n-butanol–glacial acetic acid–distilled water (60:20:20 v/v) as the solvent and the following spray reagents: 25 g $litre^{-1}$ (w/v) ninhydrin in acetone, followed by 1 g $litre^{-1}$ methyl red (w/v) in ethanol then 5 g $litre^{-1}$ (w/v) 3,5-dinitrosalicylic acid in M NaOH; 4 g $litre^{-1}$ (w/v) 2,4-dinitrophenylhydrazine in 2M HCl; 20 g $litre^{-1}$ (w/v) *p*-dimethylaminobenzaldehyde in 5M HCl; 600 g $litre^{-1}$ (w/v) perchloric acid, M HCl, 40 g $litre^{-1}$ (w/v) ammonium molybdate, distilled water (5:10:25:60 v/v); Cl_2 vapour, *o*-tolidine (saturated) in 2% (w/v) acetic acid and 0·05 N KI; modified Rydon-Smith reagent (Dawson, Elliot & others, 1969). Before spraying, the chromatograms were also examined for fluorescence after exposure to ultraviolet light at 254 nm. All the residues were also analysed for amino-acids using a Beckman Multichrom Automated Amino Acid Analyser System.

RESULTS

Location of anti-inflammatory activity in column eluate

The complete eluate from the Sephadex G 25 column (see Materials and Methods) comprised 70 portions each of 20 ml which were combined to make five separate

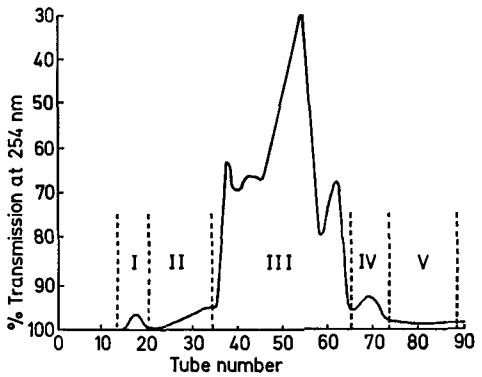

FIG. 1. Location of fractions I to V in column eluate following chromatography of Diaflo PM 10 ultrafiltrate and on Sephadex G 25.

fractions I, II, III, IV and V. A diagram of the elution pattern is given in Fig. 1 and the results of the animal testing are shown in Table 1. Fraction IV is the area reported by McArthur & others (1972) to contain L-tryptophan and significant anti-inflammatory activity was found in this portion. Anti-inflammatory activitv was also found in fraction II but fractions I and V were inactive. Fraction III could not be tested because it proved toxic to the rats. The toxic reactions comprised convulsions followed by rapid death from respiratory failure. The possibility that the anti-inflammatory activity, found in fractions II and IV, was caused by some of the fraction III toxic material being present in the other fractions and producing either a fall in the blood pressure or counter-irritancy, was investigated. The administration of fraction II or fraction IV, in concentrations which significantly reduced foot swelling in the carrageenan oedema reaction, did not affect the blood pressure of the anaesthetized rat and failed to produce a significant increase in rat paw oedema formation when tested by the technique of Atkinson, Boura & Hicks (1969). To determine if fractions II and IV either contained different anti-inflammatory substances or if they represented the beginning and end of a broad peak of activity eluted from the column, fractions II and IV from a single preparation were re-run independently on the same column. Activity was measured in eluate portions

Table 1. *Location of anti-inflammatory activity in column eluate.* Results are given as mean ± standard deviation and expressed as percentage increase in paw volume relative to 0 h. Those marked * show a statistically significant difference ($P < 0.005$) from the corresponding control animals, the numbers of animals tested are given in parentheses.

Time (h) after carrageenan injection	Control (25)	Column eluate fraction I (5)	II (25)	IV (25)	V (5)
1	30·2 ± 11·8	37·5 ± 11·8	20·2 ± 8·9*	19·9 ± 8·3*	26·8 ± 8·0
2	70·4 ± 21·2	84·3 ± 5·8	35·4 ± 18·3*	39·5 ± 16·5*	75·8 ± 19·3
3	97·3 ± 11·6	105·1 ± 11·1	56·5 ± 21·2*	61·3 ± 24·7*	96·1 ± 23·8
4	110·6 ± 14·4	114·0 ± 12·0	75·8 ± 26·1*	82·3 ± 29·7*	106·3 ± 4·5

Table 2. *Distribution of anti-inflammatory activity when separated fractions II and IV were re-run on original column.* Results expressed as in Table 1, the corresponding control value (15 animals) being 107·3 ± 8·4 at 3 h after carrageenan injection.

Original fraction	Distribution on re-running II	III	IV
II (10)	73·5 ± 16·0*	70·2 ± 16·0*	90·3 ± 10·9*
IV (5)	85·4 ± 8·4*	78·9 ± 19·3*	103·5 ± 19·4

corresponding to fractions II, III and IV and the results are given in Table 2. The anti-inflammatory activity from the separated fractions II and IV was found to spread on re-running into regions corresponding to the original fractions II, III and IV.

Passage of anti-inflammatory activity through membranes of varying pore sizes

The results in Table 3 show that significant anti-inflammatory activity appeared in fraction IV when the plasma was ultrafiltered through the following Diaflo membranes: PM 10, UM 2 and UM 05. These membranes retain spherical molecules of 10 000, 2000 and 500 molecular weight and above. Significant activity was also obtained in fraction II using the Diaflo PM 10 and UM 05 membranes. The results suggest that the anti-inflammatory substance behaves as a compound with a molecular weight of 1000 or less.

The effects of boiling with water, and acid and of exposure to proteolytic enzymes on the anti-inflammatory activity

Anti-inflammatory activity persisted after heating the residue from fraction IV either with water or HCl for 72 h at 120°. Exposure of the eluate residue to insoluble proteolytic enzymes (non-specific protease, trypsin or papain) for 24 h at 36° also resulted in no loss of activity.

Partial purification of the residue from fraction IV by solvent extraction

Anti-inflammatory activity could not be removed by repeated solvent extraction from a dried residue from fraction IV with either ethanol, acetone or chloroform. Before solvent extraction, thin-layer chromatography showed the presence of at least 30 components in the mixture. These were considerably reduced in number upon

Table 3. *Anti-inflammatory activity in ultrafiltrates of human plasma.* (The results given as in Table 1).

Time after carrageenan injection (h)	Control (20)	Ultrafiltration membrane PM 10 (5)	UM 2 (5)	UM 05 (10)
1	26·4 ± 12·2	15·2 ± 6·4	16·9 ± 5·1	18·5 ± 8·8
2	88·8 ± 17·6	34·0 ± 14·1*	37·9 ± 15·1*	47·5 ± 23·3*
3	101·6 ± 14·2	34·9 ± 18·0*	48·1 ± 9·9*	59·8 ± 31·3*
4	111·9 ± 13·8	52·6 ± 16·5*	65·9 ± 18·7*	75·4 ± 31·4*

acetone extraction. Subsequent ethanol extraction of the acetone residue reduced further the components and at this stage fewer than six spots could be seen using the Rydon-Smith reagent. No other sprays used showed any significant colour reactions at this degree of purification and no amino-acids could be detected using an amino acid analyser. Thus a substantial degree of purification of the extract could be achieved by using simple solvent extraction.

DISCUSSION

The results of the present work provide confirmation of other evidence (McArthur & others, 1972) that human serum or plasma contains a substance possessing anti-inflammatory activity in the carrageenan oedema test in the rat. In addition, they show that the substance responsible for this activity may be isolated from human plasma by simple ultrafiltration and subsequent elution through a Sephadex G 25 column as a broad peak smearing over the low molecular weight range. The activity was not due either to counter-irritant or to hypotensive effects. The results, using a range of ultrafiltration filters, provide additional data that the anti-inflammatory activity is associated with a substance of molecular weight of 1000 or less. The activity is stable towards boiling, acid hydrolysis or prolonged exposure to several proteolytic enzymes showing that it is not a linear peptide or similar structure. Partial purification was carried out by extraction of the residue of the column eluate with several organic solvents and this procedure removed a large number of components giving positive reactions with a variety of chemical spraying reagents. The only positive chemical reaction observed in the most purified preparations is that with the Rydon-Smith reagent, a non-specific test given by substances such as imines containing a C–N bond (Rydon & Smith, 1952).

There have been a number of reports describing the isolation of substances, from inflammatory exudates in man and in experimental animals, which have been designated as endogenous anti-inflammatory materials since they may inhibit the development of the acute inflammatory reaction both at the original lesion and also at any fresh sites (DiPasquale & Girerd, 1961; Billingham & Robinson, 1972). The best documented is a protein-like molecule which is apparently synthesized in the liver as a response to acute inflammatory processes and may be detected in the circulation (Billingham, Gordon & Robinson, 1971). The isolation procedures and electrophoretic characteristics of this substance are very different from the small molecule described in the present work unless the protein-like anti-inflammatory substance is a composite molecule consisting of a small active ligand attached to a carrier protein. A further difference is that the substance described in the present paper may be an integral part of a natural defensive system against inflammation normally present in the circulation rather than being produced in response to an inflammatory stimulus.

Further critical experiments are necessary. The substance responsible for the experimental anti-inflammatory activity in human plasma must be further characterized and its possible binding to one or more proteins in the circulation investigated to determine if the commonly used antirheumatic drugs exert their specific actions in the human rheumatic diseases by interacting with protein bound forms of the anti-inflammatory substance (McArthur, Dawkins & others, 1971).

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