

## THE ROLE OF BACTERIAL CONTAMINATION IN THE ISOLATION OF APPARENT ANTI-INFLAMMATORY FACTORS FROM RABBIT ANTI-LYMPHOCYTIC SERUM

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1 Rabbit anti-guinea-pig lymphocytic serum was fractionated by gel filtration to obtain partially purified materials possessing anti-inflammatory activity. The pharmacological properties of these materials were then studied.

2 Two fractions were found which reproducibly contained significant activity. One of these activities caused inflammation at the site of injection and was associated with high molecular weight protein (200,000). The other activity was found in a low molecular weight fraction but was shown to be due to small amounts of endotoxin from Gram negative bacteria. These organisms contaminated the fractions in spite of the recommended precautions for gel filtration having been taken.

3 The endotoxin-containing fraction completely abolished leucocyte infiltration into the rat foot which had been injected with kaolin. It had no apparent effect on circulating haemolytic complement. It caused maximal elevation of serum 11-hydroxycorticosteroid concentrations and was found to cause the release of pharmacologically active amines. Many of the previously reported naturally occurring anti-inflammatory substances have similar pharmacological properties to those of the endotoxin-containing fraction.

4 It was concluded that doubt will exist about the presence of anti-inflammatory factors in mammalian body fluids unless stringent precautions are taken to exclude measurable bacterial contamination.

5 These experiments also cast doubt on the validity of accepted procedures for excluding microbial growth from columns used in the fractionation of serum.

### Introduction

It has been known for many years that inflammation at one site in an animal is reduced when an inflammatory response has been previously elicited at another site (Laden, Quentin Blackwell & Fosdick, 1958). Several hypotheses have been put forward to explain this phenomenon including the postulated existence in the blood of substances with anti-inflammatory activity.

Reports have appeared in the literature describing the occurrence of anti-inflammatory activity in exudates from granuloma pouches (DiPasquale, Girerd, Beach & Steinetz, 1963; Billingham, Robinson & Robson, 1969) and in serum from rats injured by laparotomy (van Gool, Schreuder & Ladiges, 1974), in rabbit anti-lymphocytic serum (Billingham, Robinson & Gaugas, 1970) and in normal human serum (McArthur, Smith & Freeman, 1972).

In this paper we have searched for such active substances in rabbit anti-lymphocytic serum which had been fractionated under conditions where some

bacterial growth occurred in spite of standard precautions and also under conditions where bacterial contamination was reduced by stringent methods.

We have examined some of the pharmacological properties of our fractions to ascertain if they are similar to those of active fractions described by other workers.

### Methods

#### *Preparation of anti-lymphocytic serum*

Rabbit anti-guinea-pig lymphocytic serum was raised as described by Billingham, *et al.* (1970). New Zealand White rabbits (Ranch Rabbits, Copthorne, Sussex) under pentobarbitone sodium (Abbott) anaesthesia, were bled from the carotid artery through a polythene cannula into 100 ml polypropylene centrifuge tubes seven days after the final injection of

guinea-pig thymocytes. The blood was allowed to clot for at least 2 h at room temperature or occasionally overnight at 4°C before centrifugation at 2000 g for 20 minutes. The serum was freeze dried and stored at -20°C until required. It was not sterile.

Sterile anti-lymphocytic serum was prepared in a similar manner except that the rabbits were bled under aseptic conditions. The dissection area was treated with 0.5% Hibitane (ICI) in 70% ethanol. The cannulae were sterilized in a partially evacuated dessicator containing formaldehyde vapour for 16 h at room temperature and the blood was collected in autoclaved blood bottles in which clotting was permitted to take place as before. The blood was centrifuged and the serum siphoned into autoclaved bottles before freeze drying in a formaldehyde sterilized freeze drier. The serum was stored as before. No bacteria could be cultured from it.

#### *Fractionation of serum*

The fractions were separated by gel filtration on columns of Sephadex G-150 or G-25 (medium grade, Pharmacia), equilibrated with 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 200 mM NaCl, adjusted to pH 6.0 with NaOH. Experimental details of typical separations are given in the legends to Figures 1 and 2.

For separation on Sephadex G-25, the sample was applied by upward flow. The column was then inverted and elution continued by downward flow. By this inversion technique a sharper application band of the very viscous protein mixture was achieved. The column eluates were monitored at 254 nm (LKB Uvicord 1) and by electrical conductivity to detect changes in salt concentration (Radiometer CDM 2e conductivity meter equipped with a flow cell). The columns were kept flowing when not in use and were occasionally flushed with buffer containing 0.02% sodium azide, as recommended by Pharmacia (1970), in order to keep bacterial growth to a minimum. The fractions were pooled as indicated in Figures 1 and 2 and dialysed four times against at least 10 vol of deionized water, before freeze drying and subsequent testing. Columns were sterilized for some experiments by autoclaving the Sephadex, buffers and all glassware for 20 min at 121°C and leaving the plastic parts of the columns in contact with 1.0% Hycolin (William Pearson Ltd.) for 16 hours. Fractions from the sterilized columns were dialysed at 4°C to keep microbial growth to a minimum.

#### *Measurement of anti-inflammatory activity*

Anti-inflammatory activity was assessed by the rat hind paw kaolin oedema test. Groups of six male Wistar rats (Anglia Laboratory Animals) weighing 180–200 g were injected with the test sample in either 1 ml of 0.9% w/v NaCl solution (saline) given sub-

cutaneously into the adductor region or in 0.1 ml saline into the plantar region of the left hind limb. Control animals received the same volume of saline alone. Kaolin (25%, Hopkins & Williams) in 0.05 ml saline was injected 30 min later into the plantar region of the right hind paw. The volume of each paw was measured plethysmographically before and at intervals during the inflammatory response. By this method both the inflammatory and anti-inflammatory responses of the fractions could be assessed simultaneously in the same animal.

Anti-inflammatory activity was compared to that of indomethacin (Merck, Sharp & Dohme) standards injected similarly and is expressed as a potency relative to indomethacin (mg indomethacin equipotent to 1 mg freeze dried sample). Indomethacin (1.75–15 mg) was dissolved in 95% ethanol (0.5 ml), neutralized with 1M NaOH and diluted with saline to a final volume of 7 ml; 1 ml was injected subcutaneously in each rat.

#### *Preparation of rat paws for histology*

Histological examination of the rat paws was carried out 6 h after kaolin injection. The animals were killed with chloroform vapour, the paws were excised, cut longitudinally into two parts and fixed in 10% buffered formalin pH 7.0 for a minimum of 24 hours. The paws were decalcified in 15% disodium edetate (EDTA) pH 7.0 and the end point (3–4 weeks) was determined by X-ray of the specimen. They were then dehydrated through an ascending series of alcohols, cleared in xylene and embedded in Fibrowax (m.p. 56°C; Raymond A. Lamb). Sections (5 µm) were cut on a Leitz base sledge microtome and stained in Ehrlich's haematoxylin and eosin.

#### *Measurement of serum concentrations of corticosteroids and complement*

Rat blood samples were obtained, under pentobarbitone anaesthesia, by cannulation of the dorsal aorta with a Venocut winged infusion set (Argyle). The serum was prepared as for rabbit serum.

Total serum haemolytic complement was measured by the method of Kabat & Mayer (1961).

Serum 11-hydroxysteroid concentrations were determined by a modification of the method of Lowry, McMartin & Peters (1973). The steroids were extracted from the serum (2 ml) by gentle shaking with petroleum ether (boiling range 60–80°C, 6 ml) for approx. 20 minutes. After centrifugation the top layer was discarded and the lower layer (1 ml) was extracted with deionized water (2 ml) and dichloromethane (4 ml) by gentle shaking for 40 minutes. After centrifuging the lower dichloromethane layer (1 ml) was assayed for steroids by the above method.

*Depletion of pharmacologically active amines*

Rats were depleted of 5-hydroxytryptamine and histamine by injection (i.p.) of 0.12 ml per rat of 1 mg/ml compound 48/80 (Burroughs Wellcome) twice daily for 3 days and 0.24 ml of 1 mg/ml 48/80 twice on the fourth day. The rats were used 5 h after the final injection.

*Identification and culture of bacteria*

Bacteria were cultured initially in Trypticase Soy Broth (B.B.L.) incubated for approximately 18 h at 37°C or 22°C. Gram stains (Jarvis, 1973) were performed on the cultures. All secondary cultures from the broth were subcultured separately on Trypticase Soy Agar, MacConkey Agar, Cystine Lactose Electrolyte Deficient Agar and Pseudosel Agar (B.B.L.) incubated at 37°C for approximately 18 hours. Five morphologically similar colonies were subjected to standard biochemical tests and subsequently identified (Cowan, 1974). Each culture was concurrently checked for purity by culturing it on Trypticase Soy Agar.

All identified organisms were cultured in Trypticase Soy Broth at their optimum temperature for 18 h and washed three times with 0.1% peptone water by centrifugation at 6000 g for 5 min, once with deionized water and then freeze dried ready for testing for anti-inflammatory activity.

Bacterial endotoxin was detected by the *Limulus* amoebocyte lysate gelation test of Levin, Poore, Zauber & Oser (1970) as described in Sigma Technical Bulletin No. 210 (1973). The commercial endotoxin was *Escherichia coli* lipopolysaccharide type 1 from serotype 0127:88 (Sigma).

**Results***Separation of anti-inflammatory fractions*

When non-sterile anti-lymphocytic serum (specific activity, s.c., 0.004 mg indomethacin equivalents per mg) was chromatographed on a column of Sephadex G-150, two peaks were shown to be associated with anti-inflammatory activity after dialysis and freeze drying of the eluate (Figure 1, Table 1). This procedure took approximately three days and was carried out at room temperature. The anti-inflammatory activity (Table 1) in pool 2 eluted from the column with an apparent molecular weight of about 200,000 and had an activity (s.c.) of 0.01 mg indomethacin equivalents/mg in the kaolin paw oedema test. The active material in pool 5 eluted from the column with the serum salts (conductivity peak) and consequently had an apparent molecular weight of less than 5000. It was found to have an activity (s.c.) of 0.08 mg indomethacin equivalents per mg.

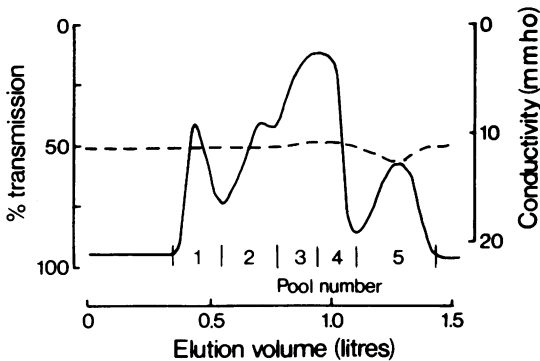
In order to fractionate the low molecular weight components with the higher specific activity more quickly and with better resolution, anti-lymphocytic serum was applied to a column of medium grade Sephadex G-25 (Figure 2). Two active fractions were again eluted (Table 1). The first eluted with all the serum proteins which are totally excluded by this gel. This corresponded to the expected behaviour of the previously observed fraction with low specific activity, having a molecular weight of 200,000. A second peak of activity eluted with an apparent molecular weight of 1400 and had a specific activity (s.c.) of 0.44 mg indomethacin equivalents per mg (Table 1). Pool 2 from the Sephadex G-150 column and the low molecular weight active fraction from the Sephadex G-25 column (pool 3) were dialysed separately against

**Table 1** Typical specific anti-inflammatory activities of pools from sterile and non-sterile Sephadex G-150 and G-25 columns

<i>Sephadex G-150</i>					<i>Sephadex G-25</i>				
<i>Pool No.</i>	<i>Non-sterile</i>		<i>Sterile</i>		<i>Pool No.</i>	<i>Non-sterile</i>		<i>Sterile</i>	
	<i>Activity/mg</i>	<i>mg/kg</i>	<i>Activity/mg</i>	<i>mg/kg</i>		<i>Activity/mg</i>	<i>mg/kg</i>	<i>Activity/mg</i>	<i>mg/kg</i>
1	NS	200	NS	40	1	0.01*	315	0.005*	350
2	0.01*	200	0.02*	160	2	NS	10	NS	15
3	NS	200	NS	220	3	0.44*	9	NS	15
4	NS	200			4	NS	10	NS	15
5	0.08*	50	NS	42					

Pools were obtained from the columns as described in Figures 1 and 2. These were assayed in the rat paw kaolin oedema test. Their specific activities by subcutaneous injection relative to indomethacin, were calculated as described in the Methods section. Units are mg indomethacin equivalent to 1 mg freeze dried sample.

\* Values significantly different ( $P < 0.05$ ) from the saline controls. NS = not significantly different from controls at the dose used.

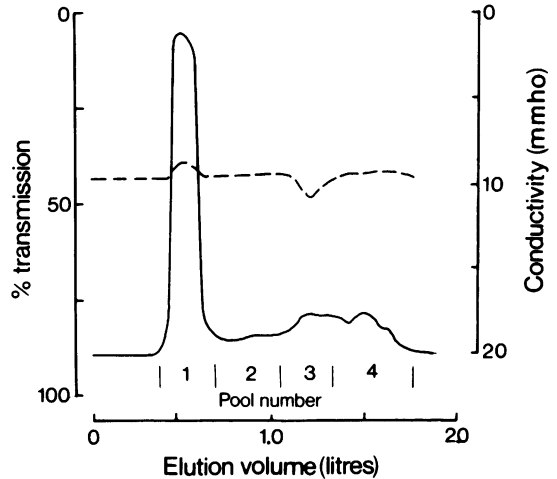


**Figure 1** A typical fractionation of rabbit anti-guinea-pig lymphocytic serum on Sephadex G-150. Freeze dried serum (3 g) was dissolved in 20 mM  $\text{NaH}_2\text{PO}_4$ , 200 mM NaCl adjusted to pH 6.0 with NaOH (25 ml) and applied to a  $4.8 \times 85$  cm column of Sephadex G-150 eluted by upward flow at approximately 60 ml/hour. The eluate was monitored at 254 nm (—) and by electrical conductivity (---). The fractions were pooled as indicated.

deionized water, freeze dried and used for further studies. The high molecular weight component from Sephadex G-150 chromatography will be referred to as fraction A and the apparently low molecular weight fraction from Sephadex G-25 chromatography as fraction B.

Fraction B did not dialyse through Visking tubing and was rechromatographed on a similar Sephadex G-25 column to ascertain whether any change had taken place. Its elution behaviour was then that of a material with a high molecular weight (greater than 5000). It was subsequently found that this active material was excluded by Sepharose 6B gel which indicates a molecular weight of greater than  $4 \times 10^6$ . This apparent change in molecular weight was investigated by taking samples of the fraction B from several columns, culturing and Gram staining them. Examination under the microscope showed the presence of Gram negative bacteria which, when subcultured, were identified as *Pseudomonas aeruginosa*, *Escherichia coli*, occasional *Enterobacter agglomerans* and an unidentified lactose-fermenting rod.

In order to assess the effects due to these bacteria, sterile rabbit anti-lymphocytic serum (activity 0.004 mg indomethacin equivalents per mg, s.c.) was chromatographed on an autoclaved column of Sephadex G-25. A similar fractionation to that shown in Figure 2 was achieved and no Gram negative bacteria could be cultured from the dialysed fractions. After freeze-drying in a sterile freeze-drier, the fractions were tested for anti-inflammatory activity. The excluded peak was found to contain low activity



**Figure 2** A typical fractionation of rabbit anti-guinea-pig lymphocytic serum on Sephadex G-25. Freeze dried serum (10 g) was dissolved in 20 mM  $\text{NaH}_2\text{PO}_4$ , 200 mM NaCl adjusted to pH 6.0 with NaOH (60 ml) and applied to the  $5.0 \times 80$  cm column of medium grade Sephadex G-25. The eluate was monitored at 254 nm (—) and by electrical conductivity (---). Fractions were pooled as indicated.

(0.005 mg indomethacin equivalents per mg, s.c.), but the low molecular weight fraction now contained no detectable activity (Table 1).

The same result was obtained when sterile anti-lymphocytic serum was chromatographed similarly on an autoclaved column of Sephadex G-150. The active fraction with a molecular weight of 200,000 (fraction A) was obtained and the low molecular weight fraction was inactive.

#### *Anti-inflammatory activity of contaminating bacteria*

Each species of contaminating organism was cultured in fluid medium, collected and washed. The freeze dried cells were tested for anti-inflammatory activity (s.c.) (Table 2). It was found that *E. coli* was highly active, *Ent. agglomerans* was less active and *Ps. aeruginosa* and the lactose-fermenting bacterium had no detectable activity. A commercial preparation of *E. coli* endotoxin was also tested for anti-inflammatory activity and found to be 120 times as active as indomethacin (w/w).

A number of samples of fraction B were, therefore, prepared by both non-sterile and sterile procedures. These were tested for both anti-inflammatory activity and for endotoxin content by the *Limulus* amoebocyte lysate gelation test (Table 3).

Since some fractions from the columns were found

**Table 2** Specific anti-inflammatory activity of the freeze dried, washed bacterial cells cultured from the column fractions.

<i>Species</i>	<i>Activity/mg</i>	<i>mg/kg</i>
<i>Escherichia coli</i>	7.24*	0.5
<i>Pseudomonas aeruginosa</i>	NS	2.5
Unidentified lactose fermenter	NS	0.5
<i>Enterobacter agglomerans</i>	1.26*	2.5

Experimental details and units are as described in Table 1.

to contain anti-inflammatory activity and others not, the growth of bacteria was measured in each pool after sterile anti-lymphocytic serum was chromatographed on a column of Sephadex G-25 as before. The fractions were pooled in the manner exemplified by

Figure 2, and 75 ml portions of each were inoculated with the arbitrary amount of  $10^5$  cells *E. coli* in 0.1% peptone (1 ml). Growth was allowed to take place at room temperature and the amount of contamination was assessed by counting viable cells and measuring the endotoxin content after 3 days. Table 4 shows that the highest concentration of both viable cells and endotoxin was found in pool 3 which corresponds to the fraction B.

*Pharmacological effects of fractions A and B*

Fraction A, prepared as in Figure 1 was found to cause a severe, prolonged local inflammation when injected into the rat food pad (Figure 3). At the dose used only a small anti-inflammatory effect was observed.

Fraction B, prepared as in Figure 2, also induced inflammation at its injection site (plantar injection) but the reaction was more transient and on the basis of the

**Table 3** Comparison of specific anti-inflammatory activity with content of bacterial endotoxin of four preparations of fraction B.

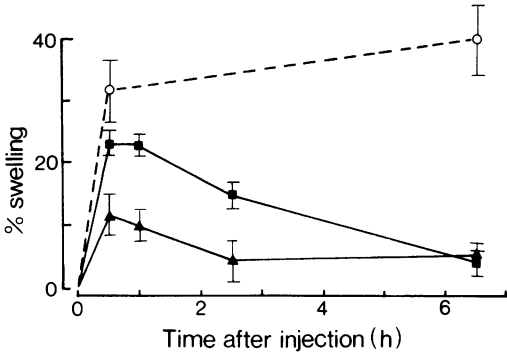
<i>Sample</i>	<i>Oedema test</i>		<i>Limulus test</i>				<i>Amount tested per 0.1 ml (µg)</i>
	<i>Activity/mg</i>	<i>mg/kg</i>	<i>Incubation time (min)</i>				
			5	10	15	20	
Non sterile	0.59*	9	+++				3.00
Non sterile	0.40*	6.5	+++				6.70
sterile	NS	11	-	+	++	+++	7.34
sterile	NS	15	-	-	+	+++	8.15

The fractions B from four columns, run under non-sterile or sterile conditions were tested (s.c.) for anti-inflammatory activity by the rat paw kaolin oedema test and for endotoxin content by the *Limulus* amoebocyte lysate gelation test. The specific anti-inflammatory activity is expressed as in Table 1. The time and degree of gelation of the *Limulus* amoebocyte extract is dependent on the amount of endotoxin and this was estimated visually at the times indicated. +++=solid gel; ++=weak gel; +=increase in viscosity or opacity. Flocculation was taken as a negative result.

**Table 4** The growth of bacteria in column fractions

<i>Pool No.</i>	<i>No. viable bacterial cells/ml</i>	<i>Limulus test</i>					
		<i>Incubation time (min)</i>					
		5	6	7	8	9	10
1	$10^3$	-	-	-	+	++	++
2	$10^5$	-	-	-	-	+	+
3	$10^8$	-	+	++	+++	+++	+++
4	$10^5$	-	-	-	-	-	+

Pools 1 to 4 were prepared as in Figure 2 under sterile conditions. Samples (75 ml) were inoculated with  $10^5$  viable washed *E. coli* (1 ml) and incubated at 20°C for 3 days. Samples were taken for bacterial cell counting and for endotoxin testing by the *Limulus* test. Endotoxin determination results are shown as in Table 3 for a 1:100 dilution of the sample. By this dilution approximately 3 µg of fraction B (pool 3) was tested.



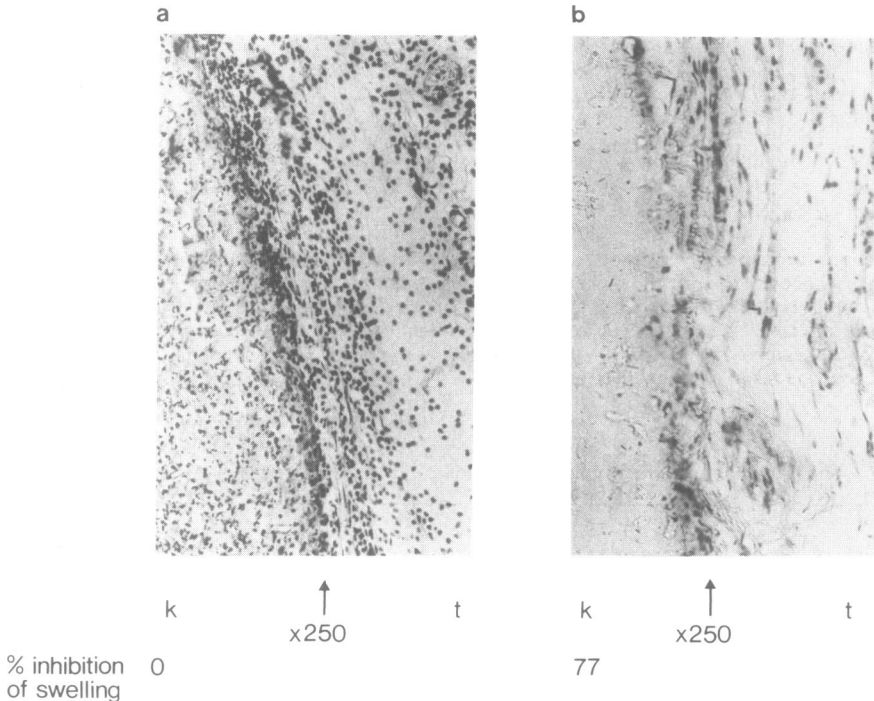
**Figure 3** The inflammatory effect of fractions A and B. Fraction A (0.1 ml, 200 mg/ml) in NaCl (O) or fraction B (0.1 ml, 10 mg/ml) (■ and ▲) were injected into the left foot pads of groups of 6 rats and the foot volumes measured at the times indicated. These are expressed as a percentage of the original volume. Vertical lines show s.e. Percentages are corrected for the volume due to 0.1 ml saline injection. One group (▲) had been pretreated with compound 48/80.

dose employed (1 mg), it had a more pronounced anti-inflammatory effect. In addition, doses which had a small but significant effect on the kaolin oedema caused no local inflammation of the injection site.

The pharmacological properties of fraction B were investigated in more detail. The local inflammation caused by fraction B was partially inhibited by pretreatment of the animals with compound 48/80 (Figure 3) and also by treatment with mepyramine maleate and methysergide bimaleate (2 mg/kg i.p.) together. Neither of these treatments antagonized the anti-inflammatory action of this fraction.

The effect of subcutaneous injection of fraction B on various pharmacological mediators was also measured. It caused a rapid increase in serum 11-hydroxycorticosteroid concentration from the untreated level of about 200 ng/ml, to 350 ng/ml which persisted for the duration of the experiment (6 hours).

Subcutaneous injection of fraction B caused no significant change in the serum haemolytic complement level of rats after 4 h, no change in the total number of circulating leucocytes and completely



**Figure 4** Photomicrographs of sections of rat paws bearing kaolin oedemas: (a) shows part of the kaolin deposit and surrounding tissue of the paw of a control rat (treated with saline); (b) shows a similar area of a rat treated with 40 mg/kg fraction B subcutaneously. Arrow in each photograph shows the boundary between the kaolin deposit (k) with surrounding tissue (t).

abolished leucocyte infiltration into the kaolin injection site (Figure 4).

## Discussion

Our results confirm that freeze dried unfractionated rabbit anti-lymphocytic serum has anti-inflammatory activity. When the serum was fractionated by gel filtration under conditions where bacterial growth was minimized only one active fraction was obtained. It caused inflammation itself and may act via a counter-irritant mechanism (Laden, *et al.*, 1958; Atkinson & Hicks, 1971) and thus may not be a true anti-inflammatory factor.

When anti-lymphocytic serum was fractionated under conditions where bacterial growth occurred in spite of standard precautions, two active fractions were found. One fraction had similar properties to fraction A. The other had an apparently low molecular weight (less than 5000). This fraction (fraction B) was not dialysable and when rechromatographed its elution properties were consistent with a high molecular weight. Fraction B was found to contain Gram negative bacteria and when these bacteria were not present on the column, the anti-inflammatory activity did not appear. Thus the activity was generated by the action of bacteria.

Each species of bacteria found was therefore tested for anti-inflammatory activity in the kaolin oedema test and it was found that *E. coli* and *Ent. agglomerans* possessed activity. The lipopolysaccharide endotoxin of the former was extremely active and it is likely that this was one of the active principles.

The bacteria grow in and contaminate mainly the low molecular weight fraction. This is not surprising since it contains amino acids, small peptides, small carbohydrates and all the serum salts. It is, therefore, a more easily assimilated medium than mixtures of large proteins.

Our results show that, in spite of the recommended laboratory precautions (Pharmacia, 1970) for the prevention of microbial growth on columns, namely by constantly keeping the buffer flowing and occasionally washing with 0.02% sodium azide, it is still possible for column fractions to become contaminated. Some of the contaminating organisms were found to grow on agar in the presence of 1% sodium azide. Even after autoclaving the Sephadex, buffer and glassware and soaking the plastic parts of the column in disinfectant, it was still possible for some contamination to occur, but from Gram positive organisms, which were probably present in the laboratory atmosphere. These organisms were, however, found to be inactive in the kaolin oedema test.

The *Limulus* amoebocyte lysate gelation test is an

extremely sensitive method for detecting the presence of bacterial endotoxin and has proved useful in our work. Concentrations of as little as 5 ng/ml can readily be detected. Unfortunately, it is too sensitive for the purposes of detecting whether a quantity of endotoxin will influence kaolin oedema formation and this necessitates large dilutions in endotoxin-free water. The commercial preparation of endotoxin which we have used has an ED<sub>50</sub> of approximately 25 µg/kg rat in the kaolin oedema test, which is nearly four orders of magnitude greater than the detection level of the *Limulus* test. The *Limulus* test is not strictly quantitative owing to the differing solubilities, the method of preparation of the endotoxin and the dependency on the species of bacteria. The rate of gelation and texture of the gel are however related to the concentration of a given endotoxin.

The shock reaction caused by endotoxin from Gram negative bacteria causes an increase in serum 11-hydroxycorticosteroid concentration (Munoz, 1961), activation of the complement system (Bladen, Gewurz & Mergenhagen, 1967) and an inhibition of leucocyte infiltration into areas of inflammation (Conti, Cluff & Scheder, 1961). Our fraction B caused both an increase in serum corticosteroid concentration and an inhibition of leucocyte infiltration. However, we found no evidence of complement depletion 4 h after injection of fraction B.

Fraction B also caused inflammation at its injection site. The rapid rate of formation and disappearance of this oedema suggested that 5-hydroxytryptamine and/or histamine were involved in this process. This hypothesis is supported by the finding that when amine release was prevented by previous administration of 48/80 or when methysergide and mepyramine were administered, the oedema was at least partially prevented. The relative contributions of histamine and 5-hydroxytryptamine were not investigated. Reduction of such oedema did not interfere with the ability of fraction B to inhibit oedema induced in the contralateral paw by injection of kaolin. Further, low doses of fraction B which did not cause measurable inflammation were still active against kaolin oedema. Therefore, the mode of action of fraction B is not through a counter-irritant mechanism.

Fraction B eluted from the Sephadex G-150 column at approximately 0.9 column volumes and has the same elution volume as the fraction C referred to by Billingham *et al.* (1970) in their fractionation of anti-lymphocytic serum. However, these workers, appear to have recycled some of the serum proteins over this peak, thus obscuring the presence of much low molecular weight material. It is, therefore, possible that the activity which they found in their fraction C is the same as that described by us as fraction B. Also, fraction B of Billingham *et al.* (1970) is probably the same as our fraction A, since these have the same elution volumes (approximately 0.4 column volumes).

Billingham *et al.* (1970) did not regard this material as being a true anti-inflammatory substance in view of its immunosuppressive and lymphocytotoxic properties.

The active material isolated from normal human serum (Ford-Hutchinson, Insley, Elliott, Sturgess & Smith, 1973) was isolated by gel filtration as two low molecular weight fractions from the edges of an optical density peak, the central portion of which could not be tested because it proved toxic to rats. An injection of bacterial endotoxin would have the same result if sufficient were injected. The human fraction appears to act by preventing leucocyte migration but not by depleting complement (Walker, Smith, Ford-Hutchinson & Billimoria, 1975).

Léme & Schapoval (1975), in their work with inflamed paw perfusates have been able to show oedema inhibiting activity when the perfusate was injected into the rats. Concomitant with this they observed an increase in serum corticosterone concentration.

None of these authors has reported taking any precautions to exclude contamination by bacteria nor have they attempted to measure any endotoxin which may have been present. The pharmacological properties of these anti-inflammatory fractions are similar to those of our endotoxin containing fraction B.

It is clear from our work that where experiments have been carried out under conditions which exclude significant bacterial contamination, no characterizable low molecular weight anti-inflammatory substances could be found in anti-lymphocytic serum.

We thank Mr A. Turnbull for doing the rat paw oedema experiments, Mr M. Heavens for the bacteriological work and helpful suggestions and discussions, Mr P. Boon for providing facilities for the bacteriology, Mr R. Christian for the microscopy and Mrs P. Rham, Superintendent Radiographer at Horsham Hospital for providing X-ray facilities.

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(Received August 23, 1976.  
Revised September 26, 1976.)