J. Pharm. Pharmac., 1976, **28**, 859–864 *Received July* 26, 1976

Some biological and pharmacological properties of inflammatory exudates

N. S. DOHERTY* AND B. V. ROBINSON†

Department of Pharmacology, Guy's Hospital Medical School, London, SE1 9RT, U.K.

Inflammatory exudates were obtained from polyester sponges which had been implanted subcutaneously in rats four days previously. This material was found to be anti-inflammatory when injected into other rats in which carrageenan pleurisy had been induced. At a dose of 600 mg kg^{-1} exudate inhibited the formation of pleural effusion, emigration of both neutrophils and mononuclear cells and the accumulation of β-glucuronidase and lactic dehydrogenase. The same dose of sponge exudate did not however inhibit the increased vascular permeability induced in the rat skin or rat foot following injection of 5-hydroxytryptamine, histamine, prostaglandin E_1, or bradykinin. Furthermore sponge exudate did not reduce the haemolytic complement titre of rat serum either *in vivo* or *in vitro*. The possible mechanism of anti-inflammatory action of exudate is discussed.

Exudates obtained from sites of inflammation in many species, including man, have been shown to be anti-inflammatory in a variety of *in vivo* tests (Robinson & Robson, 1964; Billingham, Robinson & Robson, 1969a, 1969b; Atkinson, Whittle & Hicks, 1971; Bonta & Noordhoek, 1974). It has been suggested that this activity forms part of an endogeneous system for the regulation of the inflammatory process (Billingham & others, 1969b), furthermore that disturbance in this system may take place during chronic inflammatory disease.

Although there is widespread agreement on the existance of such activity, the exact nature of the active component and the mechanism by which exudate produces its anti-inflammatory effect remain unknown. It has so far been established that activity is not detectable in normal serum, is independent of the adrenal glands (Robinson & Robson, 1964), and that the active component is a protein (Billingham & others, 1969a) which is synthesized by the liver (Billingham, Gordon & Robinson 1971).

With regard to the mechanism of action, many alternatives have been considered, including counter-irritation (Atkinson & Hicks, 1971; Billingham & Robinson, 1972) lysosomal stabilization (Capstick, Lewis & Cosh, 1975; Doherty & Robinson, 1976) and complement depletion (Bonta & Noordhoek, 1974). However none of these provide a totally satisfactory explanation of the actions of exudate, and in view of the potential importance of such a substance, the *in vivo* properties of exudate have been further investigated.

* Present address: Department of Cell Biology, Roche Products Ltd., Welwyn Garden City, Herts, AL7 3AY, U.K.
† Correspondence.

METHODS

Animals

Wistar rats maintained on standard laboratory diet and with free access to tap water, were bred in the Animal Department, Guy's Hospital Medical School with the exception of those used in the carrageenan pleurisy experiments which were obtained from Tuck & Sons.

Collection of sponge exudate

Polyester sponges (Declon 49—Declon Plastics Ltd) were implanted subcutaneously into the shaved dorsal surface of 150–200 g male rats as described by Billingham & others (1969a). Four days after implantation the animals were killed and the exudates collected by squeezing the sponges. After clotting for 1 h at 20° the exudates were centrifuged (1500 *g* for 15 min) and the supernatants dialysed overnight against 1% ammonium bicarbonate at 4°. The exudates were then freeze-dried and stored at −20°. Serum from normal rats and from sponge bearing rats was also collected, dialysed and freeze-dried in a similar manner. The exudates and sera used in these experiments were pooled samples from 10 to 20 rats.

Carrageenan pleurisy

For the study of carrageenan pleurisy it was found necessary to use Category **** rats (see MRC-LAC

Manual No. 1, 1974) in order to avoid the pulmonary infections endemic in many conventionally housed colonies. Carrageenan pleurisy was induced as described by Vinegar, Truax & Selph (1973). 0·25 ml of 0·2% w/v carrageenan (Viscarin, Marine Colloids) in 0·9% saline was injected into the pleural cavity while the animals were lightly anaesthetized with ether. At 1, 2, 4 and 8 h groups of animals were anaesthetized and blood samples collected from the inferior vena cava into heparinized syringes. The animals were then killed and 1·0 ml of 0·9% saline containing 20 U ml^{-1} heparin was injected into the pleural cavity. After massaging the thorax gently for 30 s the chest wall was opened and the effusion collected using a pasteur pipette. The tip of the pipette was covered with a short length of silicone rubber tubing to prevent the glass tip from scraping cells from the pleural membranes.

The weight of recovered fluid was determined and duplicate smears of both blood and effusion were prepared which were stained with May-Grunwald Giemsa stain. The total nucleated cell numbers were determined using an electronic particle counter (Model ZF, Coulter Electronics) having first lysed the erythrocytes. Differential white cell counts were made on the stained smears. The remainder of the blood and effusion from each animal was centrifuged (1500 *g* for 15 min) and the cell-free supernatants used for determination of enzyme levels. Lactic dehydrogenase (E.C. 11.1.27) was assayed using a commercial kit (Boehringer). β-Glucuronidase (E.C. 3.2.1.31) was assayed by the method of Brittinger, Hirschhorn & others (1968).

In one experiment, animals were given a subcutaneous injection of sponge exudate (600 mg kg^{-1} dissolved in 0·9% saline) at the same time as the carrageenan injection and were killed 4 h later. In another experiment, the animals received the first injection of sponge exudate as above, followed by a second similar injection after 4 h. The animals were then killed a total of 8 h after the carrageenan injection. Control animals received subcutaneous injections of 0·9% saline.

Carrageenan foot oedema

The technique used was that of Doherty & Robinson (1975). Freeze-dried sponge exudate or normal rat serum (600 mg kg^{-1} in 0·9% saline) was injected subcutaneously into the scruff of the neck of rats, 150 to 200 g, immediately before the sub-plantar injection of 0·1 ml of a 1·0% w/v solution of carrageenan in 0·9% saline. Foot volumes were measured at intervals thereafter using the method of Van Arman, Begany & others (1965). Results are expressed as the increase in foot volume (ml).

Vascular permeability

The effect of exudate on 5-hydroxytryptamine (5-HT) and on bradykinin-induced increases in permeability was studied using the rat foot. 40 μg of 5-HT creatinine phosphate (May & Baker) or 50 μg of bradykinin triacetate (Sandoz) prepared in 0·1 ml of 0·9% saline was injected into the subplantar surface of the rat foot and the increase in foot volume measured 60 min later using the same method as for the carrageenan oedema. Sponge exudate (600 mg kg^{-1}) or 0·9% saline was injected subcutaneously into the scruff of the neck 1 h before the subplantar injections.

The effect of exudate on histamine- and on prostaglandin E_1-(PGE_1) induced increases in permeability was studied using the dye leakage technique following intradermal injection of these substances. This method was used because of their much weaker effect on vascular permeability. Rats were injected subcutaneously into the scruff of the neck with either sponge exudate (600 mg kg^{-1} in 0·9% saline) or 0·9% saline. One h later they were injected intravenously with 0·5% w/v Evans blue (Harvey Laboratories) in 0·9% saline (4 ml kg^{-1}) followed immediately by intradermal injections of saline, PGE_1, (Upjohn) and histamine acid phosphate (BDH) into the shaved dorsal skin. Each rat received duplicate injections of 20 μl of phosphate buffered saline, histamine acid phosphate (55·2 μg in 20 μl of phosphate buffered saline) and PGE_1 (2 μg in 20 μl of phosphate buffer saline). The injections were made under halothane anaesthesia. One h later the animals were anaesthetized and blood samples taken from the inferior vena cava. They were then killed and the blue lesions excised. Evans blue was extracted from the plasma and skin samples using the method of Harada, Takeuchi & others (1971), and vascular permeability calculated as μl of plasma per lesion.

Complement assay

The total haemolytic complement titre of rat serum was determined by finding the time required for an aliquot of serum to lyse 50% of a standardized suspenion of sensitized sheep erythrocytes (Boackle & Pruitt, 1974; Lachmann, Hobart & Aston, 1973). A sample of serum was obtained from a control rat and used to construct a calibration curve. The titres of all test sera were expressed as % of this reference sample. Serial serum samples were obtained from individual rats from the retro-orbital plexus during light ether anaesthesia.

RESULTS

Carrageenan pleurisy

12 rats were killed immediately after injection of carrageenan into the pleural cavity. The mean weight of fluid recovered from the pleural cavity, including 1 ml of washing solution, was 1·155 g (s.e.m. 0·022). Since a total of 1·250 ml had been injected (0·25 ml of carrageenan solution +1·0 ml of saline), 0·095 ml of fluid had not been recovered from the pleural cavity. This amount was therefore added to all subsequent results. The weight of effusion (g) has been regarded as equivalent to the volume (ml) since its specific gravity is sufficiently close to unity to make correction unnecessary.

During the first 2 h after injection of carrageenan no effusion was formed, but between 2 and 8 h there was a rapid accumulation of fluid into the pleural cavity (Fig. 1A). The accumulation of leucocytes showed a time course similar to that of effusion formation (Fig. 1B). However, of the cells present at 4 h, only 4% were mononuclear, while at 8 h this proportion had risen to 34%. The rest of the cells were mainly neutrophils with only a small (<1%) number of mast cells in various stages of degranulation. As reported by others (Hurley, Ryan & Friedman, 1966; Velo, Dunn & others, 1973), it proved difficult to differentiate between different types of mononuclear inflammatory cell. Nevertheless the cells in these experiments appeared to be principally macrophages or blood monocytes with very few lymphocytes.

The appearance of β-glucuronidase in the effusion also followed a time course very similar to that of effusion formation, i.e. a 2 h lag period followed by a period of rapid accumulation (Fig. 1C). In contrast, lactic dehydrogenase accumulated rapidly during the first 4 h but remained constant thereafter (Fig. 1D). The dilution involved in the collection of the pleural effusion makes it difficult to calculate accurately the concentration of cells and enzymes as opposed to the total amounts. However measurement of blood concentrations in some experiments indicated that the levels in the effusion were many times higher than those in the blood.

In animals treated with exudate and killed 4 h after carrageenan injection all the parameters measured, with the exception of lactic dehydrogenase accumulation, were inhibited (Table 1A). Since at this stage of the reaction very few mononuclear cells had migrated into the pleural cavity the inhibition of leucocytes obtained (53%) was entirely due to inhibition of neutrophil accumulation. Animals killed 8 h after injection of carrageenan, which were given an injection of sponge exudate at the same time as the injection of carrageenan, were given a second injection of exudate 4 h later, because a preliminary experiment showed that a single injection produced only a small effect at 8 h. Table 1B shows that by using this procedure all parameters were inhibited significantly, including lactic dehydrogenase accumulation. Both neutrophil and mononuclear cell accumulation was inhibited by about 50%.

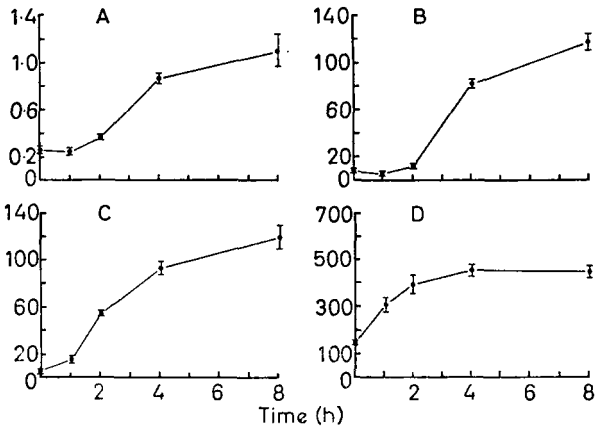

FIG. 1. The time course of carrageenan pleurisy as indicated by: A. Effusion weight (g). B. Total leucocyte count ($\times 10^6$). C. Total β-glucuronidase (U). D. Total lactic dehydrogenase (mU). Rats were killed at intervals after intrapleural injection of 0·5 mg of carrageenan in 0·25 ml of 0·9% saline, and the effusion collected from each rat. The samples were weighed and the cell concentration determined, they were then centrifuged and β-glucuronidase and lactic dehydrogenase determined in the supernatants. Results are expressed as the total amounts present in the pleural cavity. Each point is the mean ± standard error of the mean for observations from at least 5 rats.

Table 1. *The effect of sponge exudate on various aspects of the inflammation produced 4 or 8 h after intrapleural injection of carrageenan.*

	Effusion (g)	Leucocyte count ($\times 10^6$)	β-glucuronidase (U)	Lactic dehydrogenase (mU)
A				
At 4 h				
% reduction induced by sponge exudate	88	53	46	25
P value	<0·001	<0·005	<0·001	N.S.
B				
At 8 h				
% reduction induced by sponge exudate	89	52	61	75
P value	<0·001	<0·001	<0·001	<0·005

Groups of 7 rats were given an intrapleural injection of carrageenan immediately following a subcutaneous injection of either sponge exudate (600 mg kg^{-1}) or 0·9% saline. In A the rats were killed 4 h later and the effusions collected and analysed. In B the rats were treated similarly but in addition received a second injection of sponge exudate 4 h after the first and were killed 8 h after the injection of carrageenan. The effect of sponge exudate is expressed as the percentage reduction in mean value of each parameter when compared with the mean value in the saline treated control group.

Vascular permeability

Sponge exudate had no effect on the increased vascular permeability induced by any of the four mediators tested.

Complement

Following force dialysis and freeze-drying, both sponge exudate and rat serum possessed very low levels of haemolytic complement activity, and when these materials were incubated at 37° for 30 min with fresh serum, they did not reduce the haemolytic activity of the fresh serum.

Complement levels measured in freshly collected sponge exudates and in the sera of sponge bearing rats were much higher than in freeze-dried samples. Sera from sponge bearing rats collected 4 days after sponge implantation had 28% higher haemolytic complement activity ($P < 0·01$) than serum from control rats whilst the sponge exudates collected at the same time had much lower activity (60% less than normal rat serum, $P < 0·005$).

Serum complement titres were also measured before and at intervals after subplantar injection of carrageenan in animals treated with either sponge exudate or normal rat serum (600 mg kg^{-1} subcutaneously into the scruff of the neck). As can be seen from Table 2, sponge exudate significantly inhibited foot oedema but the slight depression of complement titre obtained was not significantly different from that produced by normal rat serum. Neither carrageenan foot oedema alone nor the subcutaneous administration of 0·9% saline produced any change in haemolytic complement titre.

Table 2. *The effect of sponge exudate on serum haemolytic complement titre during carrageenan-induced foot oedema in the rat.*

A. Haemolytic complement titre as % of initial values* at

Treatment	2·5 h	4·5 h
Sponge exudate	66·7 ± 5·8†	70·9 ± 5·1†
Normal rat serum	78·9 ± 5·5	92·7 ± 8·7

B. Foot volume measurements: Increase in foot volume (ml)*

Treatment	1 h	2 h	3 h	4 h
Sponge exudate	0·12 ± 0·02	0·26 ± 0·03	0·36 ± 0·05	0·20 ± 0·04
Normal rat serum	0·24 ± 0·03	0·66 ± 0·05	0·68 ± 0·04	0·43 ± 0·05
P value	<0·01	<0·001	<0·001	<0·005

Animals were treated with sponge exudate or normal rat serum (600 mg k^{-1} subcutaneously) followed immediately by an injection of carrageenan into the right hind foot. Blood samples for complement measurement were taken before treating the animals and at 2·5 and 4·5 h after injection.

A. Shows the haemolytic complement titre in both groups expressed as a percentage of the initial values. The values in the exudate treated group (†) were not significantly different from those in the corresponding serum treated group.

B. Shows the increase foot volumes (ml) in both groups at hourly intervals.

* All results are the mean of six observations ± standard error of the mean.

DISCUSSION

The time course of fluid accumulation and leucocyte migration during carrageenan pleurisy was similar to that obtained by Vinegar & others (1973). These authors did not measure enzyme concentrations but in our experiments β-glucuronidase followed a similar pattern of appearance to leucocytes and effusion. Only lactic dehydrogenase showed a distinctly different picture reaching a peak much earlier in the reaction. The most likely explanation for the early release of lactic dehydrogenase is that during the first 4 h following injection of carrageenan the plasma membranes of the endogenous cell population are sufficiently damaged by either the trauma of the injection or the toxicity of carrageenan to permit the leakage of soluble cytoplasmic enzymes without the concomitant release of lysosomal enzymes. A similar phenomenon has been observed in experimentally damaged rabbit skin (Boyles, Lewis & Westcott, 1970). The appearance of lysosomal enzymes at a later stage suggests that they are derived from the invading inflammatory cells coming either from lysosomal disruption following phagocytosis of the carrageenan (Zurier, Hoffstein & Weissman, 1973; Allison, Harington & Birbeck, 1966) or from specific secretion induced by the presence of carrageenan (Allison & Davies, 1975).

It was hoped that exudate might have demonstrated a selective inhibition of one of the parameters of the pleurisy enabling its site of action to be identified. Di Rosa, Sorrentino & Parente (1972), for example,

on the basis of similar evidence, have concluded that the primary site of action of the non-steroidal anti-inflammatory drugs is inhibition of mononuclear cell emigration, although it has been contested (Ford-Hutchinson, Smith & others, 1975; Blackham & Owen, 1975). The results of the carrageenan pleurisy experiments in which exudate was used unfortunately do not permit us to draw conclusions about the mechanism of its anti-inflammatory action since exudate proved capable of inhibiting not only effusion formation but also the accumulation of enzymes and the appearance of both cell types. Nevertheless the permeability experiments indicate that it is unlikely to be due to inhibiton of mediator-induced increases in permeability. Exudate did not inhibit the effect produced by any of the important mediators which were studied, and although an action on another mediator is possible this is unlikely to be of major significance in the mechanism of action of an anti-inflammatory substance.

It is, nevertheless, possible that exudate could affect generation or release of mediators and one way in which inflammatory mediators can be generated is by activation of the complement cascade (Vogt, 1974). However, when tested *in vitro*, sponge exudate did not inhibit activation of the classical complement sequence nor did injection of sponge exudate produce a significant reduction in the circulating haemolytic complement titre. Such effects as were found were slight, transient and did not correlate with the anti-inflammatory potency of different batches of sponge exudate. Since it has been found that even the low levels of complement which remain after depletion of complement with cobra venom factor are sufficient to permit a normal inflammatory reaction (Wiener, Lendvai & others, 1973) it seems unlikely that sponge exudate is acting through complement depletion.

These results are in contrast to those of Bonta & Noordhoek (1974) who showed a marked reduction in serum haemolytic complement titres following injection into rats of anti-inflammatory material obtained from 4 day old carrageenan granulomas. Their anti-inflammatory factor also reduced complement titres *in vitro*. This material may however still contain small amounts of carrageenan which can itself reduce complement titres both *in vivo* and *in vitro* (Borsos, Rapp & Crisler, 1965; Davies, 1963; & Doherty, 1974).

Despite the absence of an inhibitory effect with sponge exudates on haemolytic complement activity it is still possible that sponge exudate interacts with components in the complement system which are important mediators of the inflammatory response but are not limiting for immune lysis. Such an interaction would not be detected by the experiments described in this paper. Walker, Smith & others (1975), for example, found that an anti-inflammatory plasma factor inhibits the generation of chemotactic activity which takes place when complement is activated but does not inhibit immune lysis. Further experiments are required to determine whether this is also the case with sponge exudates.

Acknowledgements

We wish to thank the Arthritis and Rheumatism Council for financial support, Mr Ken Biggins for technical assistance, and the Upjohn Pharmaceutical Company for the prostaglandin.

REFERENCES

ALLISON, A. C. & DAVIES, P. (1975). In: *Mononuclear Phagocytes in Immunity, infection and pathology*, 487–506. Editor: Van Furth, R. Oxford: Blackwell.

ALLISON, A. C., HARINGTON, J. S. & BIRBECK, M. (1966). *J. exp. Med.*, **124**, 141–154.

ATKINSON, D. C. & HICKS, R. (1971). *Br. J. Pharmac.*, **41**, 480–487.

ATKINSON, D. C., WHITTLE, B. A. & HICKS, R. (1971). *Eur. J. Pharmac.*, **16**, 254–256.

BILLINGHAM, M. E. J., GORDON, A. H. & ROBINSON, B. V. (1971). *Nature New Biology*, **231**, 26–27.

BILLINGHAM, M. E. J. & ROBINSON, B. V. (1972). *Br. J. Pharmac.*, **44**, 317–320.

BILLINGHAM, M. E. J., ROBINSON, B. V. & ROBSON, J. M. (1969a). *Ibid.*, **35**, 543–557.

BILLINGHAM, M. E. J., ROBINSON, B. V. & ROBSON, J. M. (1969b). *Br. med. J.*, **2**, 93–96.

BLACKHAM, A. & OWEN, R. T. (1975). *J. Pharm. Pharmac.*, **27**, 201–202.

BOACKLE, R. J. & PRUITT, K. M. (1974). In: *Protides of the Biological Fluids*. Proceedings of the twenty-first colloquium. Brugge 1973. 613–617. Editor: Peeters, H., London: Pergamon.

BONTA, I. L. & NOORDHOEK, J. (1974). *Experientia*, **30**, 419–422.

BORSOS, T., RAPP, H. J. & CRISLER, C. (1965). *J. Immun.*, **94**, 662–666.

BOYLES, S., LEWIS, G. P. & WESTCOTT, B. (1970). *Br. J. Pharmac.*, **39**, 798–808.

BRITTINGER, G., HIRSCHHORN, R., DOUGLAS, S. D. & WEISSMANN, G. (1968). *J. Cell Biol.*, **37**, 394–411.

CAPSTICK, R. B., LEWIS, D. A. & COSH, J. A. (1975). *Ann. rheum. Dis.*, **34**, 213–218.

DAVIES, G. E. (1963). *Immunology*, **6**, 561–568.

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

DOHERTY, N. S. (1974). Further studies on the anti-inflammatory activity found in inflammatory exudates, 109–110. Ph.D. Thesis, University of London.

DOHERTY, N. S. & ROBINSON, B. V. (1975). *J. Pharm. Pharmac.*, **27**, 701–703.

DOHERTY, N. S. & ROBINSON, B. V. (1976). *Biochem. Pharmac.*, **25**, 2039–2044.

FORD-HUTCHINSON, A. W., SMITH, M. J. H., ELLIOT, P. N. C., BOLAM, J. P., WALKER, J. R., LOBO, A. A., BADCOCK, J. K., COLLEDGE, A. J. & BILLIMORIA, F. J. (1975). *J. Pharm. Pharmac.*, **27**, 106–112.

HARADA, M., TAKEUCHI, M., FUKAO, T. & KATAGIRI, K. (1971). *Ibid.*, **23**, 218–219.

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

LACHMANN, P. J., HOBART, M. J. & ASTON W. P. (1973). *Complement technology* in Handbook of experimental immunology. Editor: Weir, D. M. 2nd edn, Vol. 1, Chap. 5, London: Blackwell.

ROBINSON, B. V. & ROBSON, J. M. (1964). *Br. J. Pharmac.*, **23**, 420–430.

VAN ARMAN, C. G., BEGANY, A. J., MILLER, L. M. & PLESS, H. H. (1965). *J. Pharmac. exp. Ther.*, **150**, 328–334.

VELO, G. P., DUNN, C. J., GIROUD, J. P., TIMSIT, J. & WILLOUGHBY, D. A. (1973). *J. Path.*, **111**, 149–158.

VINEGAR, R., TRUAX, J. F. & SELPH, J. L. (1973). *Proc. Soc. exp. Biol. Med.*, **143**, 711–714.

VOGT, W. (1974). *Pharmac. Rev.*, **26**, 125–169.

WALKER, J. R., SMITH, M. J. H., FORD-HUTCHINSON, A. W. & BILLIMORIA, F. J. (1975). *Nature, Lond.*, **254**, 444–446.

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

ZURIER, R. B., HOFFSTEIN, S. & WEISSMANN, G. (1973). *Arthritis Rheum.*, **16**, 606–618.