Br. J. Pharmac. (1975), 53, 85-91

THE POSSIBLE OCCURRENCE OF ENDOGENOUS ANTI-INFLAMMATORY SUBSTANCES IN THE BLOOD OF INJURED RATS

D.C. ATKINSON

Department of Pharmacology, Pharmaceutical Research Laboratories, Reckitt & Colman Pharmaceutical Division, Hull HU8 7DS

R. HICKS

Postgraduate School of Studies in Pharmacology, University of Bradford, Bradford BD7 1DP

1 Using the carrageenin rat paw oedema test as an assay, an attempt has been made to confirm the presence of anti-inflammatory activity in the blood of rats with a chronic inflammatory lesion induced by a polyester sponge, and to relate such activity to the systemic anti-inflammatory effect exerted *in situ* by the lesion. In addition, plasma from rats given acetic acid intraperitoneally has been examined for anti-inflammatory properties.

2 The activity of serum obtained from sponge-bearing adrenalectomized Wistar rats did not differ significantly from that obtained from animals without implants. Furthermore, implanted sponges exerted no systemic anti-inflammatory effect.

3 Similar experiments were performed in sponge-bearing adrenalectomized Sprague-Dawley rats, plasma instead of serum being examined for anti-inflammatory activity. The plasma removed from such animals possessed no anti-inflammatory activity, whilst implanted sponges exerted a small, yet significant, systemic inhibitory effect.

4 When the irritancy of sponge implants was augmented with croton oil, more marked systemic anti-inflammatory effects were observed. However, the plasma obtained from rats injured in this manner exerted no anti-inflammatory effect.

5 No anti-inflammatory activity could be detected in plasma samples obtained from rats treated with doses of acetic acid capable of producing pronounced systemic anti-inflammatory effects.

6 It was concluded that the induction of these inflammatory lesions in rats does not appear to lead to a detectable release of endogenous anti-inflammatory substances into the circulation.

Introduction

The hypothesis that irritants exert their systemic anti-inflammatory action by releasing into the blood stream a factor or factors capable of inhibiting inflammation at distant sites is supported by observations that the blood of some injured rats possessed anti-inflammatory properties (Laden, Blackwell & Fosdick, 1958. Robinson & Robson, 1964; Billingham, Robinson & Robson, 1969). Furthermore, Goldstein, Schemano, Demeo & Beiler (1967) found in parabiotic rats that the systemic anti-inflammatory activity of intraperitoneally administered kaolin was effected via the blood.

As part of a study designed to elucidate the mechanisms involved in the systemic anti-inflammatory actions of irritants, some of the evidence favouring a humoral mechanism of action has been re-examined. In particular, attempts have been made to confirm the presence of anti-inflammatory activity, detected by Robinson & Robson (1964) and Billingham *et al.* (1969) in the blood of rats bearing a chronic inflammatory lesion caused by the subcutaneous implantation of polyester sponges, and to relate such activity to the systemic anti-inflammatory effects exerted *in situ* by implanted sponges. In addition, the plasma removed from rats acutely injured by acetic acid has been examined for anti-inflammatory properties.

Methods

Animals

In attempts to verify the experiments of Billingham *et al.* (1969) Wistar rats (A.S.L.) were

used; in other experiments, Sprague-Dawley rats were employed for both blood sampling and evaluation of anti-inflammatory activity. All animals were males weighing 120-255 g.

Bilateral adrenalectomy was performed through a dorsal midline incision, under ether anaesthesia. The skin wound was closed with Michel suture clips. Operated animals were maintained on Spillers' Laboratory Small Animal (Autoclaved) diet and 0.9% w/v NaCl solution (saline) until they were required for further experimentation on the fourth day after the operation.

In some animals, polyester sponges (50 x 20 x 7 mm) were implanted subcutaneously, one per rat, via a dorsal skin incision (in adrenalectomized rats, this was performed immediately following the operation).

Collection and preparation of plasma and serum

Animals were anaesthetized with ether, the abdominal cavity was opened and blood was withdrawn from the inferior vena cava, using a blood sampler (Peel-A-Way Scientific, South El Monte, California) fitted with a No. O serum needle. For plasma collection, the sampler tube had previously been rinsed with heparin solution (500 u/ml) and the needle deadspace was filled with this solution at the time of sampling. Blood samples were cooled and centrifuged at approximately 800 g for 40 min at 0-5° C and the plasma removed. Overtly haemolysed samples were rejected. Plasmas from rats receiving the same pretreatment were pooled, freeze-dried and stored below 0° C. Serum samples were obtained similarly but the sampler contained no anticoagulant. Following withdrawal, the blood was transferred to a glass centrifuge tube, cooled in ice, and then centrifuged at approximately 800 *g* for 15 min at 0-5° C. Pooled sera were then poured into lengths of dialysis tubing and dialysed against 4 litre batches of 0.9% w/v NaCl solution (saline) for approximately 64 h at 2-4° C (saline batches were changed about every 24 h). The contents of the dialysis sacs were then freeze-dried and stored below 0° C. When required for use, freeze-dried plasma or serum was dissolved in water (Water for Injection, B.P.). Unless otherwise stated, the doses of plasma or serum stated in **Results** refer to the amount of freeze-dried material injected per kg.

Evaluation of anti-inflammatory activity

The anti-inflammatory activity of serum samples obtained from Wistar rats was determined in rats of the same strain using the carrageenin rat paw oedema test (Winter, Risley & Nuss, 1962) in a manner similar to that employed by Billingham *et al.* (1969). The samples were administered s.c. immediately following the subplantar carrageenin injection (0.1 ml of a 1% w/v suspension in 0.9% w/v saline; increase in left hind paw volume measured 5 h later). Control animals received a similar dose volume (10 ml/kg) of 0.9% w/v saline. Food and water were withdrawn immediately before the start of the experiment.

Systemic anti-inflammatory activity in Wistar rats with implanted sponges was determined 4 days after implantation by injecting the left hind paw of each rat with 0.1 ml of a 1% w/v carrageenin suspension. The subsequent increase in paw volume was measured 5 h later. The oedema obtained in sponge-bearing rats was expressed as a percentage of that obtained in appropriate control animals. Food and water were withdrawn immediately before the start of the experiment.

The activity of plasma samples obtained from Sprague-Dawley rats was determined in similar rats using the carrageenin paw oedema method described by Atkinson, Boura & Hicks (1969). The samples were administered either i.p or s.c. 1 h before the subplantar injection of carrageenin (0.05 ml of a 1% w/v suspension); the increase in left hind paw volume was measured 3 h later. Control animals received a similar dose volume (10 ml/kg) of the vehicle.

Systemic anti-inflammatory activity in Sprague-Dawley rats with implanted sponges was determined by injecting into their paws 0.05 ml of a 1% w/v carrageenin suspension and measuring the oedema 3 h later

The statistical significance of differences between test groups was calculated using the two-tailed Student's *t*-test. Results were considered significant if $P \leqslant 0.05$.

Results

Degree of anti-inflammatory activity induced in sponge-implanted adrenalectomized rats

In attempts to verify the experiments of Billingham *et al.* (1969), serum was collected from adrenalectomized Wistar rats bearing implanted sponges on the fourth day after the operation. The anti-inflammatory activity of prepared pooled samples was investigated and compared with that of a similar dose (600 mg/kg) of serum from rats without sponges. The results (Table 1) showed that the sera from rats, both with and without implants, produced a significant anti-inflammatory effect when compared with saline-treated rats. However, the degree of activity produced by each serum sample did not differ significantly.

To ascertain the systemic influence of sponge implantation *in vivo*, inhibition of carrageenin

oedema was determined directly in adrenalectomized sponge-bearing rats as described above; adrenalectomized rats without sponges were used as controls. The results (Table 2) showed that the implanted sponges exerted no significant anti-inflammatory effect.

In similar experiments with Sprague-Dawley rats, plasma, but not serum, was collected from donor rats 4 days after sponge implantation. Anti-inflammatory activity was evaluated by the method of Atkinson *et al.* (1969).

Under these conditions neither control nor test plasma samples exerted significant anti-inflammatory activity when doses of 600 and 1000 mg/kg were injected i.p. and s.c., respectively (Table 1). However, sponge implantation exerted *in vivo* a small but significant systemic anti-inflammatory effect (Table 2).

Anti-inflammatory activity exerted by sponge implantation and croton oil in adrenalectomized and in intact Sprague-Dawley rats

As the failure to detect significant anti-inflammatory activity in serum or plasma from sponge-bearing rats was associated with only weak systemic activity, it was possible that the implanted sponges were not producing a

Table 1 Anti-inflammatory effect of serum or plasma obtained from adrenalectomized rats 4 days after the subcutaneous implantation of polyester sponges, as measured against carrageenin-induced rat paw oedema

Treatment	*Dose*	*Mean increase in paw volume ± s.e. mean (ml)*	*% Inhibition of controls*	P *vs. controls*	P *vs. donor control serum/plasma*
Wistar rats*					
0.9% w/v saline (controls)	10 ml/kg, s.c.	0.83 ± 0.05†			
Non-implanted rat serum	600 mg/kg, s.c.	0.70 ± 0.04	17	<0.05	
Implanted rat serum	600 mg/kg, s.c.	0.61 ± 0.05	26	<0.01	NS
Sprague-Dawley rats**					
(a) Water for Injection, B.P. (controls)	10 ml/kg, i.p.	0.57 ± 0.04			
Non-implanted rat plasma (donor controls)	600 mg/kg, i.p.	0.62 ± 0.05	–	NS	
Implanted rat plasma	600 mg/kg, i.p.	0.58 ± 0.04	–	NS	NS
(b) Water for Injection, B.P. (controls)	10 ml/kg, s.c.	0.54 ± 0.04			
Non-implanted rat plasma (donor controls)	1000 mg/kg, s.c.	0.58 ± 0.05	–	NS	
Implanted rat plasma	1000 mg/kg, s.c.	0.61 ± 0.05	–	NS	NS

Each result is the mean of 10 observations (†9). N.S. = not significant ($P > 0.05$).
* Paw volume increases measured 5 h after subplantar injection of 0.1 ml of 1% w/v carrageenin suspension.
** Paw volume increases measured 3 h after subplantar injection of 0.05 ml of 1% w/v carrageenin suspension.

Table 2 Systemic anti-inflammatory effect 4 days after subcutaneous implantation of polyester sponges into adrenalectomized rats, as measured against carrageenin-induced paw oedema

Pretreatment	*Mean increase in paw volume ± s.e. mean (ml)*	*% Inhibition of controls*	P *vs. controls*
Wistar rats*			
Adrenalectomy alone (controls)	0.98 ± 0.05		
Adrenalectomy + sponge implanted	0.92 ± 0.05	6	NS
Sprague-Dawley rats**			
Adrenalectomy alone (controls)	0.62 ± 0.03		
Adrenalectomy + sponge implant	0.51 ± 0.03	19	<0.02

Each result is the mean of 10 observations. NS = not significant ($P > 0.05$).
* Paw volume increases measured 5 h after subplantar injection of 0.1 ml of 1% w/v carrageenin suspension.
** Paw volume increases measured 3 h after subplantar injection of 0.05 ml of 1% w/v carrageenin suspension.

sufficiently powerful irritant stimulus. To test this possibility, the sponge-induced inflammation was augmented by the s.c. injection of 0.1 ml of croton oil in close proximity to the sponge immediately following implantation.

On the fourth day after treatment, either plasma samples were obtained or systemic anti-inflammatory effects were measured. The results showed that the combination of sponge implantation and croton oil injection exerted a marked systemic anti-inflammatory effect in adrenalectomized rats (Table 3). However, only 6 out of 20 croton oil-treated rats survived the 4-day post-operative period, and 4 of the survivors were moribund by the end of the experiment. It was therefore possible that reduced oedema could have been due mainly to the poor health of the animals rather than a genuine influence of the irritancy. No attempt was made to harvest plasma from these rats because large numbers of animals would be required to obtain adequate volumes of pooled plasma. The experiments were therefore repeated in intact rather than in adrenalectomized rats; control animals received a dorsal skin incision only. Systemic anti-inflammatory activity was determined 4 days after treatment as before. Table 3 shows that systemic activity was less than that observed in adrenalectomized animals but was apparently greater than that induced by implanted sponges alone. All animals survived the entire experimental period. Plasma obtained from such animals 4 days after treatment exhibited no significant anti-inflammatory activity (Table 3).

Activity of plasma from acetic acid-treated rats

An attempt to detect anti-inflammatory activity in the plasma of rats with acute inflammation was made using a dose of acetic acid previously shown (Atkinson, 1971) to produce a marked systemic inhibition of carrageenin oedema. Plasma samples were obtained from Sprague-Dawley rats 4 h after i.p. injection of either acetic acid (60 mg/kg, as 10 ml/kg of a 0.6% v/v solution) or Water for Injection, B.P. (10 ml/kg). Table 4 shows that there was no significant anti-inflammatory activity in either control or test plasma, even at doses of 1.2 g/kg. The result was confirmed with a much higher dose of acetic acid (200 mg/kg, i.p.) which produces an approximately 85% inhibition of carrageenin oedema (Atkinson, 1971). Plasma was obtained from such animals 4 h after injection and examined within a few hours of collection. No anti-inflammatory activity was detected in either control or test plasma samples (Table 4).

Table 3 Anti-inflammatory effects of subcutaneous polyester sponges and croton oil (0.1 ml, s.c.) in adrenalectomized and intact Sprague-Dawley rats, as measured against carrageenin-induced rat paw oedema

(1) *Systemic activity (assessed 4 days after treatment)*

Pretreatment	*Mean increase in paw volume ± s.e. mean (ml)**	*% Inhibition of controls*	P *vs controls*
(a) Adrenalectomized rats			
Adrenalectomy alone (controls)	0.74 ± 0.02		
Sponge implant + croton oil	0.38 ± 0.06†	49	<0.001
(b) Intact rats			
Dorsal skin incision alone (controls)	0.61 ± 0.04		
Sponge implant + croton oil	0.46 ± 0.03	24	<0.005

(2) *Activity of plasma (collected 4 days after treatment—dose 1200 mg/kg, s.c.)*

Treatment	*Mean increase in paw volume ± s.e. mean (ml)**	*% Inhibition of controls*	P *vs. controls*	P *vs. donor control plasma*
Water for Injection, B.P., 10 ml/kg, s.c. (controls)	0.65 ± 0.04			
Plasma from intact rats with dorsal skin incisions (donor controls)	0.59 ± 0.03	11	NS	
Plasma from intact rats with sponge implant + croton oil	0.60 ± 0.03	8	NS	NS

Each result is the mean of 10 observations (†6). NS = not significant ($P > 0.05$).
* Measured 3 h after subplantar injection of 0.05 ml of a 1% w/v carrageenin suspension.

Table 4 Anti-inflammatory effect of plasma obtained from Sprague-Dawley rats 4 h after treatment with acetic acid, as measured against carrageenin-induced rat paw oedema

Treatment	*Dose (i.p.)*	*Mean increase in paw volume ± s.e. mean (ml)**	*% Inhibition of controls*	*P vs. controls*	*P vs. normal donor plasma*
(1) Freeze-dried plasma from rats given acetic acid, 60 mg/kg, i.p.					
(a) Water for Injection, B.P. (controls)	10 ml/kg	0.54 ± 0.06			
Normal donor plasma	600 mg/kg	0.44 ± 0.03	19	NS	
Acetic acid donor plasma	600 mg/kg	0.49 ± 0.04	10	NS	NS
(b) Water for Injection, B.P. (controls)	10 ml/kg	0.53 ± 0.04†			
Normal donor plasma	1200 mg/kg	0.47 ± 0.04†	12	NS	
Acetic acid donor plasma	1200 mg/kg	0.45 ± 0.02†	16	NS	NS
(2) Fresh plasma from rats given acetic acid, 200 mg/kg, i.p.					
Water for Injection, B.P. (controls)	10 ml/kg	0.46 ± 0.03			
Normal donor plasma	10 ml/kg	0.41 ± 0.03	12	NS	
Acetic acid donor plasma	10 ml/kg	0.54 ± 0.04	–	NS	–

Each result is the mean of 5 observations (†10). NS = not significant ($P > 0.05$).
* Measured 3 h after subplantar injection of 0.05 ml of a 1% w/v carrageenin suspension.

Discussion

In the present investigation, it was not possible, under a variety of conditions, to confirm the occurrence of anti-inflammatory activity in the blood of injured rats. Robinson & Robson (1964) showed that the plasma obtained from adrenalectomized rats with implanted sponges exerted anti-inflammatory activity, measured against cotton pellet granuloma and that an implanted sponge reduced the amount of granulation tissue deposited in the same animal. Billingham *et al.* (1969) also demonstrated marked anti-inflammatory activity in the serum from sponge-bearing rats when this was assayed on carrageenin oedema. However, these authors did not ascertain whether this activity was associated with a systemic effect against carrageenin oedema in similarly-treated animals. In the present investigation, the activity of serum from sponge-bearing rats, obtained from the same source as the animals used by Billingham *et al.* (1969), did not differ significantly from that obtained from animals without implants. Moreover, implanted sponges evoked no demonstrable systemic anti-inflammatory activity in this strain of rat. These results suggest that there was no detectable release into the circulation of an endogenous anti-inflammatory factor and raise the possibility that the serum activity observed by Billingham *et al.* (1969) may have been due to an artefact. There was also no significant anti-inflammatory activity detected in the plasma of sponge-bearing Sprague-Dawley rats although a small, but significant systemic effect was demonstrated in such animals.

These results are consistent with our earlier contention that sponge implantation does not lead to the accumulation of endogenous anti-inflammatory factors in the local sponge exudate (Atkinson *et al.*, 1969; Atkinson & Hicks, 1971; Atkinson, Whittle & Hicks, 1971), as suggested by Billingham *et al.* (1969).

An alternative explanation for the lack of systemic and hence blood-borne activity observed in these experiments is that the particular sponges used were not sufficiently irritant to generate the anti-inflammatory factor when implanted in either Wistar or Sprague-Dawley rats. However, the sponges were essentially the same as those used by Billingham *et al.* (1969). When the sponge-induced inflammation in adrenalectomized Sprague-Dawley rats was augmented by injecting croton oil, the systemic anti-inflammatory activity was considerably boosted. This applied to a lesser extent also to intact rats with sponge and croton oil-induced inflammation. However, the plasma removed from such rats possessed little or no anti-inflammatory activity. DiPasquale, Girerd, Beach & Steinetz (1963) also showed that serum

obtained from rats with croton oil-induced granuloma pouches did not exert significant anti-inflammatory effects. It would thus appear that the systemic influence of the severe inflammation used in the present experiments was not associated with a humoral mediator capable of suppressing carrageenin oedema.

In contrast to the previous experiments, very pronounced systemic anti-inflammatory effects can be induced acutely in rats treated with acetic acid (Atkinson, 1971). Yet, no anti-inflammatory activity could be detected in the plasma of these rats. In contrast, Laden *et al.* (1958) reported that blood taken from rats treated with another corrosive irritant, silver nitrate, possessed anti-inflammatory properties, but no supporting data have since been published.

The conflict between the present results and those of Billingham *et al.* (1969) may be possibly resolved by a consideration of the control experiments performed in the respective investigations. In the present study, the control sera and plasma were obtained from adrenalectomized animals, whereas in that of Billingham *et al.* (1969), sera from normal unoperated rats were used. It could be argued that adrenalectomy and its attendant trauma initiates some systemic anti-inflammatory effect, perhaps mediated via a humoral factor, thus masking or at least reducing any possible difference between control and test sera or plasma. In contrast, it would be easier to detect a significant difference between sera obtained from normal rats and those from adrenalectomized sponge-implanted rats. However, adrenalectomy tends to potentiate rather than inhibit carrageenin oedema, at least when assessed against sham-operated controls (Atkinson, 1972), which renders the above explanation unlikely.

Even the more severe inflammatory lesions used in the present study did not appear to lead to the release of endogenous anti-inflammatory substances into the circulation. If the marked serum activity observed by Billingham *et al.* (1969) was due to a humoral substance, then the present results suggest that it does not occur consistently and hence may be of little physiological significance. It is more likely that counter-irritant effects generally depend upon competition between inflammatory lesions for mediators or their precursors, a possibility which is supported by numerous studies (Garcia Leme, Schapoval & Rocha e Silva, 1967; Willoughby, Coote & Turk, 1969; Giroud & Timsit, 1970; Atkinson, 1971; Briseid, Arntzen & Dyrud, 1971).

The authors would like to thank Mr M.H. Smith for his assistance in the preparation of freeze-dried materials.

References

ATKINSON, D.C. (1971). A comparison of the systemic anti-inflammatory activity of three different irritants in the rat. *Archs int. Pharmacodyn. Thér.*, **193**, 391-396.

ATKINSON, D.C. (1972). Studies on the anti-inflammatory activity of irritants. Ph.D. thesis, University of Bradford.

ATKINSON, D.C., BOURA, A.L.A. & HICKS, R. (1969). Observations on the pharmacological properties of inflammatory exudate. *Eur. J. Pharmac.*, **8**, 348-353.

ATKINSON, D.C. & HICKS, R. (1971). Relationship between the anti-inflammatory and irritant properties of inflammatory exudate. *Br. J. Pharmac.*, **41**, 480-487.

ATKINSON, D.C., WHITTLE, B.A. & HICKS, R. (1971). A further observation on the anti-inflammatory activity of inflammatory exudate. *Eur. J. Pharmac.*, **16**, 254-256.

BILLINGHAM, M.E.J., ROBINSON, B.V. & ROBSON, J.M. (1969). Partial purification of the anti-inflammatory factor(s) in inflammatory exudate. *Br. J. Pharmac.*, **35**, 543-557.

BRISEID, K., ARNTZEN, F.C. & DYRUD, O.K. (1971). Inhibition of carrageenin-induced rat paw oedema by substances causing a reduction of kininogen and prekallikrein in plasma. *Acta pharmac. tox.*, **29**, 265-274.

DIPASQUALE, G., GIRERD, R.J., BEACH, V.L. & STEINETZ, B.G. (1963). Antiphlogistic action of granuloma pouch exudates in intact or adrenalectomized rats. *Am. J. Physiol.*, **205**, 1080-1082.

GARCIA LEME, J., SCHAPOVAL, E.E.S. & ROCHA E SILVA, M. (1967). Factors influencing the development of local swelling induced in the rat's paw by macromolecular compounds and heating. In: *International Symposium on Vaso-active Polypeptides: Bradykinin and Related Kinins, Ribeirão Préto, 1966*, ed. Rocha e Silva, M. & Rothschild, H.A., pp. 213-221. Sao Paulo: Edart.

GIROUD, J.-P. & TIMSIT, J. (1970). Activite anticomplémentaire et anti-inflammatoire du 48/80. *C.r. Séanc. Soc. Biol.*, **164**, 2219-2224.

GOLDSTEIN, S., SHEMANO, I., DEMEO, R. & BEILER, J.M. (1967). Anti-inflammatory activity of several irritants in three models of experimental inflammation in rats. *Archs int. Pharmacodyn. Thér.*, **167**, 39-53.

LADEN, C., BLACKWELL, R.Q. & FOSDICK, L.S. (1958). Anti-inflammatory effects of counterirritants. *Am. J. Physiol.*, **195**, 712-718.

ROBINSON, B.V. & ROBSON, J.M. (1964). Production of an anti-inflammatory substance at a site of inflammation. *Br. J. Pharmac. Chemother.*, **23**, 420-432.

WILLOUGHBY, D.A., COOTE, E. & TURK, J.L. (1969).

Complement in acute inflammation. *J. Path.*, **97**, 295-305.

WINTER, C.A., RISLEY, E.A. & NUSS, G.W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. exp. Biol. Med.*, **111**, 544-547.

(Revised May 23, 1974)