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LYSOSOMAL STABILIZATION AS THE POSSIBLE MECHANISM OF ACTION OF AN ENDOGENOUS ANTI-INFLAMMATORY PROTEIN

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Abstract—Inflammatory exudate obtained from polyester sponges implanted subcutaneously in rats is known to contain an anti-inflammatory protein. The effect of anti-inflammatory exudate on the rate of release of β-glucuronidase and acid phosphatase from isolated rat liver lysosomes has been studied. It was found that although dialysed normal rat serum greatly enhanced the release of enzymes, similarly prepared sponge exudate had very little effect. There appeared to be an inverse relationship between the ability of the various protein fractions to induce enzyme release and their anti-inflammatory activity. However, since the exudates did not reduce the rate of enzyme release below control levels it was not possible to determine whether they contained a lysosome stabilizing factor, or if they were simply less lytic than normal rat sera. Fractionation of the exudates on Sephadex G 150 did not resolve this question. The results are discussed in relation to other proteins said to contain stabilizing activity.

It has been shown previously that inflammatory exudates obtained by implantation of polyester sponges in rats demonstrate anti-inflammatory activity when reinjected into other rats [1–6] and that the active component is protein [3]. Reports in the literature suggest that one mechanism by which such proteins may produce anti-inflammatory effects is by interaction with lysosomal membranes. For example Hempel, Fernandez and Persellin [7] reported that serum from pregnant women stabilized rat liver lysosomes *in vitro*, and subsequently showed that such sera have anti-inflammatory activity in the rat [8]. Similarly, serum from rats with adjuvant arthritis has been shown by some workers [9] to possess lysosomal stabilizing activity and by others [10] to be anti-inflammatory. It is also recognized that stabilization of lysosomes may explain part of the action of anti-rheumatic drugs [11–13] although some workers dispute this [14–16].

This paper describes experiments to investigate the possibility that inflammatory exudates may exert their anti-inflammatory action by stabilization of lysosomes.

METHODS

Male Wistar rats weighing 150–250 g were used. They were maintained on standard laboratory diet and allowed water freely throughout the experiments.

Preparation of inflammatory exudate and serum. The method is essentially similar to that described previously [3]. Polyester sponges 2.5 × 5.0 × 1.0 cm (Declon 49, Declon Plastics Ltd.) were implanted subcutaneously (s.c.) into the shaved backs of rats during light ether anaesthesia. Four days later the animals were again anaesthetized with ether and blood withdrawn from the posterior vena cava to provide serum. The animals were then killed and exudate collected by squeezing the sponges. After clotting at room temperature for 1 hr the pooled samples of exudate and of serum were centrifuged at 1500 *g* for 20 min, and the cell-free supernatants filtered through a 0.45 μm Millipore filter. Finally they were force-dialysed overnight at 4° against 1% (w/v) ammonium bicarbonate and freeze-dried. The dry materials were stored in screw top bottles at −20° until required. Freeze-dried normal serum was prepared in a similar manner.

Preparation of lysosomes. Rat livers were rinsed in ice-cold 0.25 M sucrose buffered with 0.05 M Tris acetate (pH 7.4), the two largest lobes chopped into small pieces and a 10% homogenate prepared using an MRC glass homogenizer. Cellular debris and larger particles were removed by centrifuging at 600 *g* for 10 min at 4° and the supernatant, containing the suspended lysosomes, retained. This suspension was centrifuged at 25,000 *g* for 10 min at 4°, the supernatant discarded, and the pellet resuspended in buffered sucrose by gentle homogenization. Following centrifugation at 25,000 *g* for a further 10 min at 4°, the lysosomal-rich pellet was finally resuspended in buffered sucrose so that each gram of liver gave 2.5 ml of suspension.

Measurement of lysosomal stabilization. Two incubation media were used. In the first (Method A) 1 ml of lysosome suspension was added to 3 ml of 0.25 M sucrose containing 0.05 M Tris acetate (pH 7.4), and in the second (Method B), 0.5 ml of lysosome suspension was added to 4.5 ml of Hank's balanced salt solution (Wellcome) containing 10 mg/ml bovine serum albumin (Sigma) (pH 7.4). Both incubations were performed at 37° with shaking; aliquots of the reaction mixture were removed at intervals, centrifuged at 30,000 *g* for 10 min at 4° and the supernatants assayed for acid phosphatase [17] and β-glucuronidase [18].

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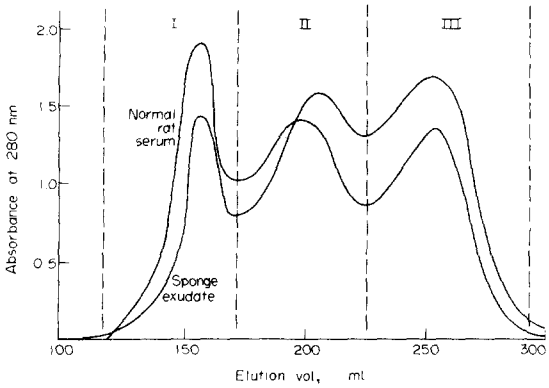

Fig. 1. Elution diagram showing the distribution of protein following separation of normal rat serum and sponge exudate on Sephadex G 150. Two hundred mg of each of the proteins was applied to a 2.5 × 1000-cm column of Sephadex G 150, and eluted with 0.5 M sodium chloride containing 0.2% sodium azide. The separated proteins were pooled as indicated (I, II, III), dialysed, freeze dried, and then tested for their effect on the rate of release of enzyme from isolated liver lysosomes.

The total enzyme activity of samples was determined following treatment with Triton-X-100 (0.2%, v/v). Freeze-dried sponge exudate or rat sera were dissolved in the test media prior to addition of the lysosome suspension, and since all these preparations possess enzyme activity this was assayed and subtracted from subsequent measurements. Hydrocortisone (B.P.) was dissolved in dimethylsulphoxide (B.D.H. Chemicals Ltd.) and added to the media prior to addition of lysosomes. The final concentration of dimethyl sulphoxide was always 1%.

Fractionation of rat proteins. Sponge exudate and normal rat serum were fractionated by gel filtration on Sephadex G 150 in a similar manner to that previously described [19]. Eight ml of a solution containing 25 mg/ml of freeze-dried protein in 0.5 M NaCl and 0.02% sodium azide was applied to a 25 × 1000-cm column at 4° and the column eluted at a rate of 13 ml/hr. The protein content of the eluant was monitored continuously by measuring its extinction at 280 nm using a flow through cell connected to a Uvicord II Ultraviolet Absorbtiometer (LKB Ltd). Samples were collected automatically every 15 min. Three protein peaks were obtained (Fig. 1); the samples from each peak were pooled, and the volumes and extinctions at 280 nm of each pool noted in order to calculate the relative proportions of each peak in the original material. The samples were then force-dialysed, freeze-dried and stored at −20° until required. The fractionated pooled materials were tested for their effects of lysosomes using Method B. Unfractionated material was shown to be effective in this system at 8 mg/ml, therefore the fractions were tested at concentrations at which they would have been present in an 8 mg/ml solution. In addition, the three fractions were recombined in the appropriate proportions and the effect of these reconstituted preparations compared with the effects of the unfractionated materials.

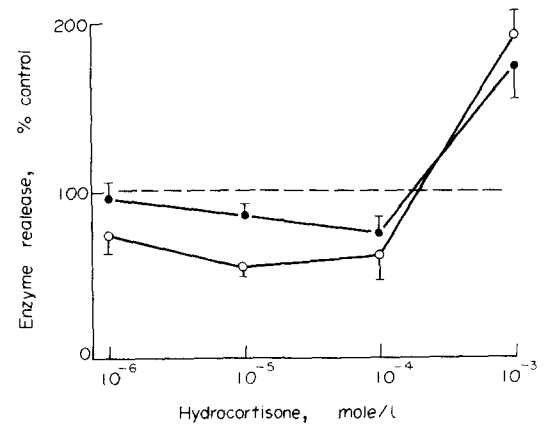

Fig. 2. The effect of increasing concentrations of hydrocortisone (M) on the amount of enzyme released from isolated liver lysosomes (incubated in isotonic sucrose buffer pH 7.4 for 60 min at 37°) expressed as a percentage of the amount of enzyme released in control incubations (without hydrocortisone): ○—acid phosphatase; ●—β-glucuronidase.

RESULTS

Neither hydrocortisone nor any of the proteins under study interfered with the enzyme assays. However, all the proteins possessed intrinsic β-glucuronidase and acid phosphatase activity which had to be taken into account when calculating the release of enzymes from the lysosomes.

Using the buffered isotonic sucrose medium (Method A) only between 1 and 3 per cent of the total enzyme activity was released in 1 hr in the control incubation. At these very low rates of release hydrocortisone demonstrated a biphasic action, increasing enzyme release from the lysosomes at 10^{-3} M but reducing it at concentrations below this (Fig. 2). The effect of the rat protein solutions was more complex (Table 1). All the preparations increased the rate of appearance of β-glucuronidase into the incubation medium and all but exudate consistently increased the rate of release of acid phosphatase. All preparations of sponge exudate caused a much slower rate of release of acid phosphatase than the other proteins, and although one preparation

Table 1. The effect of sponge exudate, serum from sponge bearing animals (inflamed serum) and normal rat serum on the release of acid phosphatase and β-glucuronidase from isolated rat liver lysosomes, incubated in isotonic sucrose (pH 7.4) at 37° for 60 min

Treatment	Enzyme activity in supernatant (% of total enzyme activity)*	
	β-glucuronidase†	acid phosphatase†
Sponge exudate (10 mg/ml)	9.3 ± 1.2	0.5 ± 0.3
Inflamed serum (10 mg/ml)	5.2 ± 0.9	3.5 ± 0.7
Normal rat serum (10 mg/ml)	14.9 ± 2.9	5.1 ± 0.3
Control	1.2 ± 0.5	2.1 ± 0.3

* Total available enzyme activity was determined in samples treated with Triton-X-100 (0.2%, v/v).

† Results are the mean of three experiments ± S.E.M.

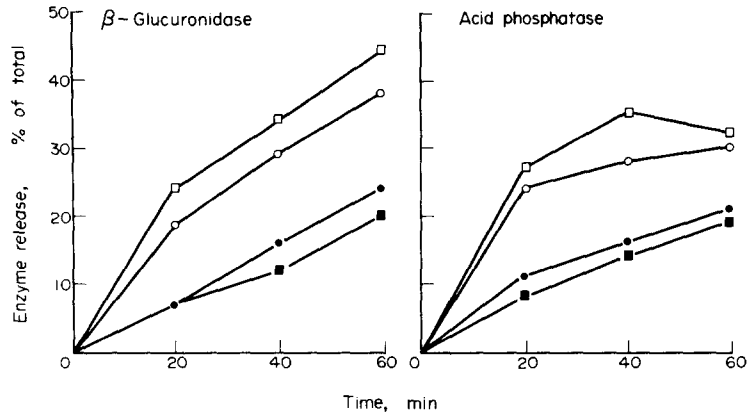

Fig. 3. A comparison of the effect of exudate (●) normal rat serum (□), serum from sponge-bearing rats (○) and control medium (■) on the rate of release of β-glucuronidase and acid phosphatase from isolated rat liver lysosomes. Incubations were performed in Hank's B.S.S. containing 10 mg/ml bovine serum albumin at 37°, and test proteins were added at a concentration of 8 mg/ml. The results are expressed as a percentage of the total enzyme which can be released by treatment of the lysosomes with Triton-X-100 (0.2‰, v/v).

reduced the rate of release below control values, with most preparations the rate of release was still faster than the control.

Using Method B, approximately 20 per cent of the total enzyme activity was released within 1 hr in the control incubations. However, hydrocortisone never inhibited the release of enzymes in this system, despite repetition of the experiment on a number of occasions varying the parameters which are thought to be critical. For example, ethanol was used as the solvent in place of dimethylsulphoxide, the water-soluble salt hydrocortisone sodium succinate was used, the enzymes were assayed using different substrates (β-*d*-glycerol phosphate for acid phosphatase and phenolphthalein glucuronide for β-glucuronidase) and lysosomes were prepared from rats which had been deprived of food overnight. In none of the experiments was the rate of enzyme release inhibited, although at 10^{-3} M enzyme release was increased in all of the experiments.

At a concentration of 8 mg/ml both normal rat serum and serum from inflamed animals increased the rate of enzyme release in system B (Fig. 3), while the effect of sponge exudate was very little different from that of the controls. When different concentrations of the proteins were used however, a slight (13 per cent) reduction in the rate of release of acid phosphatase was seen with sponge exudate at 2 mg/ml (Fig. 4).

Since sponge exudate has been shown to have its most potent anti-inflammatory effect when collected

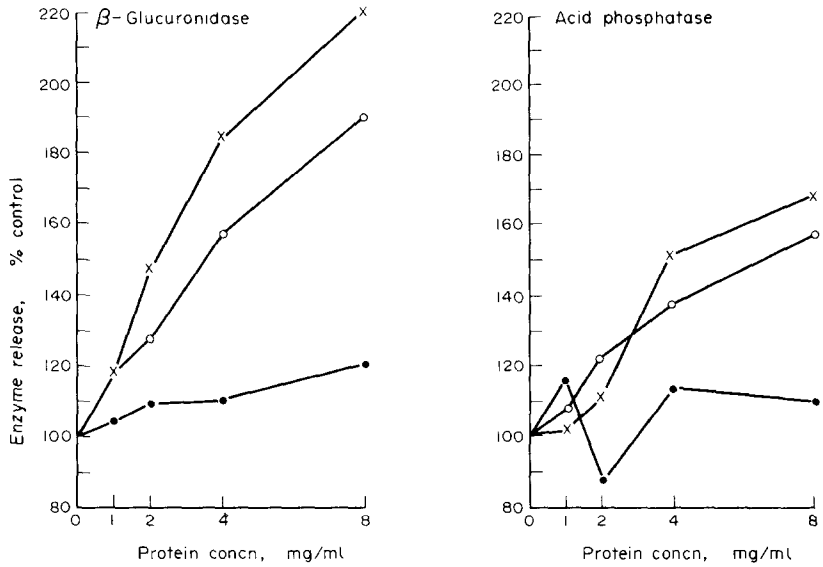

Fig. 4. The effect of increasing concentrations of exudate (●), normal rat serum (×) and serum from sponge-bearing rats (○) on the amount of β-glucuronidase and acid phosphatase released from isolated rat liver lysosomes. Incubations were performed in Hank's B.S.S. containing 10 mg/ml bovine serum albumin at 37° for 60 min. The results are expressed as a percentage of the enzyme released in control incubations over the same period.

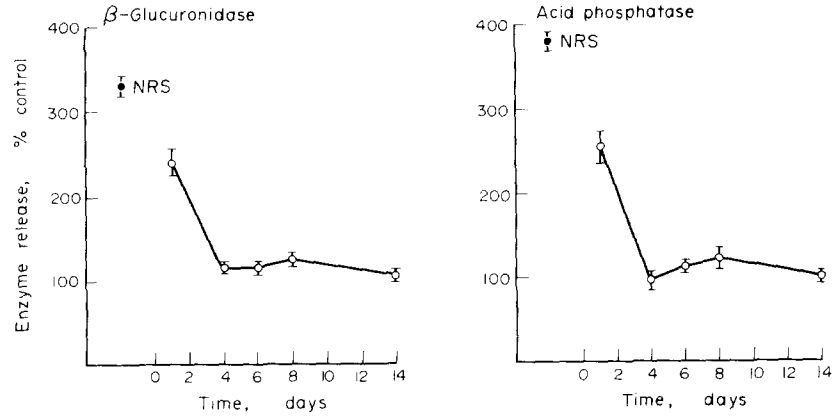

Fig. 5. The effect of normal rat serum (●) and of exudate collected on different days after sponge implantation (○) on the amount of β-glucuronidase and acid phosphate released from isolated rat liver lysosomes. Incubations were carried out for 60 min at 37° in Hank's B.S.S. containing 10 mg/ml bovine serum albumin. The results are expressed as a percentage of the enzyme released in control incubations over the same period. The figures for normal rat serum are the means of 8 observations ±S.E.M., whilst the figures for the exudate samples are the means of 4 results ±S.E.M.

Table 2. A comparison of the rate of release of β-glucuronidase from isolated rat liver lysosomes (a) obtained from normal animals, and (b) obtained from rats which had had a sponge implanted subcutaneously seven days previously

Source of lysosomes	β-Glucuronidase activity in supernatant (% of total enzyme activity)†		
	30 min	60 min	90 min
Normal rats*	14.1 ± 1.3	27.0 ± 2.9	32.0 ± 3.0
Sponge-bearing rats*	14.1 ± 1.6	25.0 ± 2.2	31.0 ± 2.6

The incubations were performed at 37° in Hank's B.S.S. containing 10 mg/ml bovine serum albumin.

* There were 8 rats in each treatment group, and the results are the mean ±S.E.M.

† Total available enzyme activity was determined by treating samples with Triton-X-100 (0.2% v/v).

4 days after sponge implantation, the relative effects on lysosomal stability of exudates collected on various days were compared. Figure 5 shows that with all the sponge exudate samples the rate of enzyme release was below that induced by normal rat serum. With the exception of the intermediate effect produced on day 1, the action of sponge exudate collected on days 4, 6, 8 and 14 did not differ significantly from the effect produced in the control incubations.

The stability of liver lysosomes from rats in which sponges had been implanted 4 days previously was compared with the stability of control lysosomes. Table 2 shows that no differences were apparent in the rate of release of enzymes from these preparations.

In an attempt to separate any labilizing and stabilizing activities in sponge exudate, and to determine whether the latter if it appeared, was in a similar fraction to the anti-inflammatory activity, fractionation of sponge exudate and normal rat serum was carried out on Sephadex G150. The activity of the three protein peaks obtained (Fig. 1) was tested using System B. The three fractions of sponge exudate were used at concentrations equivalent to their relative proportions in an 8 mg/ml solution, and Table 3 compares the rates of lysosomal enzyme release obtained with each fraction of exudate with the corresponding fraction of normal rat serum. The table also shows the rates of enzyme release for unfractionated exudate and normal rat serum and for exudate and normal rat serum which had been reconstituted from the fractionated material. In the case of exudate there was good agreement between these two figures, indicating that the majority of the activity had been recovered. This was not found to be true of the normal rat

Table 3. The effect of fractions of sponge-exudate and normal rat serum following separation of G 150 Sephadex on β-glucuronidase release from isolated rat liver lysosomes (incubated for 60 min in Hank's B.S.S. containing 10 mg/ml bovine serum albumin at 37°

Treatment	β-Glucuronidase activity in supernatant (% of total enzyme activity)†				
	Unfractionated 8 mg/ml	Fraction* I	Fraction* II	Fraction* III	Fraction* I + II + III
Sponge exudate	17.9	1.0	10.0	3.3	15.6
Normal rat serum	59.2	−5.2	25.7	17.2	30.3

* The fractions were dissolved in incubation medium at the concentrations at which they would be present in unfractionated material at 8 mg/ml.

† Total available enzyme activity was determined by treating samples with Triton-X-100 (0.2% v/v).

serum, and it is assumed that some of its labilizing activity had been lost during fractionation. It was noted that, unlike sponge exudate, the freeze-dried fractions of serum following separation did not dissolve completely, and this may explain the discrepancy.

DISCUSSION

The purpose of the present investigation was to determine whether the anti-inflammatory activity detected in sponge exudate from the rat could be attributed to a stabilizing effect of lysosomal membranes. This possibility has been investigated by assessing the effect of sponge exudate on the release of enzymes from lysosomes incubated *in vitro*. At first sight the results suggest that this may be the case since the rate of release of enzymes from lysosomes when incubated with sponge exudate was found to be less than when lysosomes were incubated with normal rat serum, especially when Method B was used. Furthermore, serum from sponge bearing animals produced an effect intermediate between the effects of sponge exudate and normal rat serum. These findings correlate with the previously established anti-inflammatory potencies of these preparations (sponge exudate > serum from sponge bearing rats > normal rat serum [1]). In addition, sponge exudate obtained on the first day after sponge implantation and which is not anti-inflammatory [4] produced a rate of enzyme release which was only slightly less than that produced by normal rat serum; whereas exudate which was collected after 4 days when its anti-inflammatory activity is maximal [4] produced minimal enzyme release (see Fig. 5). However, the failure of sponge exudate to reduce consistently the rate of enzyme release below that obtained in control incubations calls into question what is actually meant by the term "stabilization" in this system. The differences observed between the effects of sponge exudate and normal rat serum could be explained by either: the presence of lytic factors in normal serum which are not present in sponge exudates, or, the presence of stabilizing factors in sponge exudates which are not present in normal serum. In order to be able to claim that sponge exudate contains true stabilizing activity it would be necessry to demonstrate that it reduces the rate of enzyme release below that obtained in control incubations; comparison with normal rat serum is insufficient. The same criticiam applies to the "stabilizing" activities found in serum from rats suffering from adjuvant arthritis [9] and in serum from pregnant women [7]. It is noteworthy that both labilizing and stabilizing activity has been found in synovial fluids from rheumatoid arthritis patients [20].

When lysosomes were incubated in isotonic buffered sucrose, only a small proportion of their total available enzyme activity was liberated during control incubations (1 to 3 per cent). Although this system showed that hydrocortisone could stabilize lysosomes, it was thought that a more severe stress which resulted in a greater release of lysosomal enzymes would permit demonstration of stabilization with sponge exudates. Therefore the technique of Ignarro [11], Method B of this paper, was adopted. This system resulted in the release of approximately 20% of the total available enzyme activity in 1 hr. With this technique the differences between sponge exudates and normal rat serum were more pronounced, but still true stabilization was not found. However, hydrocortisone also failed to show stabilizing activity in this system. The literature contains many studies on the effects of anti-inflammatory drugs on lysosomes. In some of these stabilization has been demonstrated [11–13] whilst in others labilization or no effect has been shown [14–16]. It would therefore seem that some, as yet unidentified, technical point is critical in determining the nature of the effect produced [21].

In an attempt to separate the stabilizing and labilizing activities present in the exudate and serum samples, they were fractionated and the effects of the fractions on lysosomes studied. However none of the fractions reduced the rate of enzyme release below control levels. Nevertheless since the fraction containing anti-inflammatory activity (fraction III [2]) is still impure, further purification may yet reveal true stabilizing activity. In conclusion, it can be said that there is a very marked difference in the manner in which sponge exudate and normal rat sera interact with isolated lysosomes, and that the difference correlates inversely with the anti-inflammatory potencies of these preparations. However, because of the technical limitations of the techniques employed it is not possible to conclude that the sponge exudates contain true stabilizing activity. These results indicate that caution must be applied in the interpretation of studies of the effects of impure protein preparations on the stability of lysosomal membranes. It is suggested that studies on the rates of release of lysosomal enzymes from intact phagocytic cells [22, 23] may provide more meaningful information on the mode of action of anti-inflammatory agents than the widely used system of isolated liver lysosomes.

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