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The control of rheumatic disease by endogenous protein

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Inflammation is a feature of the rheumatic diseases and is treated clinically by the use of anti-inflammatory drugs. Unfortunately, the adverse side effects of these drugs often limit their use, particularly in the long term therapy required in chronic rheumatic disease. Consequently some workers are exploring alternative methods of therapy such as boosting the body's natural defences against the degenerative changes induced by inflammatory disease. One possible defence mechanism is that the body produces circulating proteins which can inhibit the action of inflammatory mediators produced by disease.

It has been known for some time that inflammatory exudates contain substances that have anti-inflammatory properties [1]. Billingham and Robinson [2] isolated protein from an inflammatory exudate produced by sponge implantation in the rat and found that it was capable of inhibiting carrageenin-induced inflammation in the rat by 55 per cent at a dose level of 8 mg/kg body wt when administered by subcutaneous injection.

In previous work [3] we have reported the presence of high mol. wt. proteins in human rheumatoid synovial fluid which have properties against inflammatory mediators *in vitro* and which may inhibit inflammation *in vivo*. In this communication we have isolated the high mol. wt. globulin fraction from normal rat plasma by diluting the plasma with an equal volume of 4M ammonium sulphate at 0°. The precipitated globulins were solubilised by dialysis against 0.15 M sodium chloride at 4°. The precipitation and dialysis processes were repeated three times and the final globulin preparation was freeze-dried. The preparation was tested for anti-inflammatory activity against adjuvant arthritis in the rat [4] and carrageenin induced inflammation [5] in the rat.

Male rats, Wistar strain, body wt 200 250 g, were injected intraperitoneally with either 1 ml of the reconstituted protein preparation (20 mg/kg body wt), or 1 ml of 0.15 M saline, 24 hr before the injection of adjuvant and then daily until the end of the experiment. The adjuvant (heat-killed finely ground human tubercle bacilli, kindly supplied by the Ministry of Agriculture Laboratories at Weybridge, 5 mg/ml in liquid paraffin) (0.05 ml) was injected intradermally into the foot-pad of the right hind foot. The joint diameters of both hind feet were measured in the anaesthetised animals with a micrometer.

In the carrageenin experiments the protein (10 mg/ml) was mixed with the carrageenin solution 20 mg/ml and 0.05 ml injected intradermally into the right hind footpad. Other rats were injected separately with either the protein or carrageenin alone. Inflammation was determined by measuring the volume of the injected foot immediately prior to the injection by immersing it to the hairline in a mercury bath connected to a pressure transducer linked to a pressure inducer linked to a Devices recorder [6]. Further measurements were made at 3 and 6 hr after the initial injections.

The results of the adjuvant experiment are plotted in Fig. 1. Clearly, the protein has delayed the development of arthritis in both the injected and non-injected feet. The experiment was terminated after 14 days due to two deaths in the adjuvant non-protein treated group. In a later experiment using a smaller amount of adjuvant (0.02 ml) to determine whether joint diameters in treated and untreated rats were the same of maximum values the joint swellings reached maximum values on the 22nd day. In the protein treated group the increase in joint diameters, over initial values, were 12 per cent (injected foot) and 42 per cent (non-injected foot) less than the corresponding mean values obtained in the non-protein treated group.

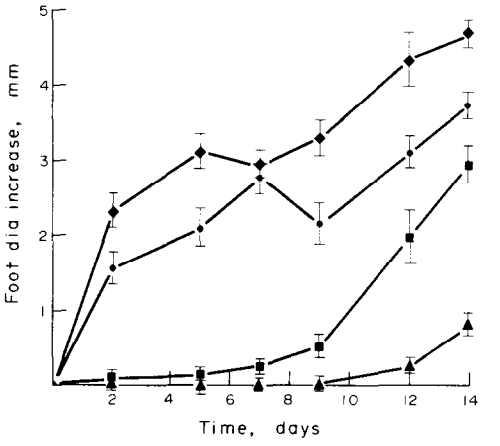

Fig. 1. Effect of high mol. wt. plasma proteins on adjuvant-induced arthritis in the rat. The results are the mean values ± S.E.M. from ten determinations. Right foot, ◆ adjuvant alone, ● adjuvant and protein. Left foot, ■ adjuvant alone, ▲ adjuvant and protein

A similar anti-inflammatory action was found in the carrageenin experiments (Table 1). Since the carrageenin and the protein were administered together it is unlikely that the anti-inflammatory action can be explained by a counter-irritant hypothesis [7]. The slight irritant action when the protein was administered alone may have been due to slight traces of denatured protein present in the preparation when mixed with the carrageenin. The protein (2 mg/kg body wt) was effective in suppressing the carrageenin-induced inflammation by about 30 per cent (Table 1) after 3 hr ($P < 0.01$).

The nature of the anti-inflammatory proteins in the preparation is not clear. In previous work, we have shown that the high mol. wt. fraction is enriched with globulins which act against lysosomal and other proteases and also with α/β globulins capable of stabilising lysosomal membranes [3]. Persellin [8] has reported that an α-globulin capable of stabilising lysosomes is present in the blood of adjuvant arthritic rats. In preliminary experiments we have found that the present globulin preparation is both capable of stabilising isolated lysosomes [3] and inhibiting

Table 1. Effect of plasma proteins on carrageenin-induced inflammation in the rat

Treatment	% increase in volume after the initial injection. (Initial values adjusted to 100) 3 hr	6 hr
Carrageenin alone	170 ± 7	144 ± 5
Protein alone	116 ± 2	120 ± 2
Carrageenin mixed with protein	140 ± 5	140 ± 4

The results are the mean values ± S.E.M. for ten determinations.

proteases [9]. The trypsin-inhibiting capacity (T.I.C.) of normal male rat blood was found to be 3.5–3.8 μmole/min/ml (Parrott, unpublished results) and the T.I.C. value of the daily globulin dose administered to the adjuvant arthritic rats was approximately equivalent to 5 ml of rat blood.

It has been suggested that protective substances against inflammation are synthetised as a result of the inflammation. Certainly certain conditions e.g pregnancy [10], viral hepatitis [11] result in the remission of symptoms in rheumatoid arthritis. Liver injury in the rat, induced by the administration of dimethylnitrososamine [12], also leads to a remission of adjuvant-induced arthritis. When other rats with adjuvant arthritis were treated with saline extracts of the livers from animals treated with dimethylnitrososamine a similar anti-inflammatory action was observed. It was suggested that the damaged livers produced substances with anti-inflammatory properties as a result of the damage since saline extracts of normal livers did not possess anti-inflammatory properties [12].

The protective substances present in the proteins used in our experiments were from normal rat plasma, and it may well be that protective substances are present in blood but are elevated as a consequence of inflammatory disease. During inflammatory disease inflammatory mediators are also produced and the course of the disease might depend on the resulting balance of inflammatory and non-inflammatory factors.

In other experiments we treated rats daily by intraperitoneal injection with 1 ml of our protein preparation (40 mg/kg body wt) for 30 days and we observed no ill effects. The major organs of the animals appeared normal on post-mortem examination.

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REFERENCES

1. T. H. Rindani, *Indian J. med. Res.* **44**, 673 (1956).
2. M. E. J. Billingham and B. V. Robinson, *Br. J. Pharmac.* **44**, 317 (1972).
3. R. B. Capstick, D. A. Lewis and J. A. Cosh, *Ann. rheum. Dis.* **34**, 213 (1975).
4. G. M. Pearson and F. D. Wood, *Arth. Rheum.* **2**, 440 (1959).
5. C. A. Winter, E. A. Risley and G. W. Russ, *Proc. Soc. exp. biol. Med.* **111**, 544 (1962).
6. D. P. Parrott and D. A. Lewis, *Biochem. Pharmac.* **24**, 925 (1975).
7. C. Laden, R. Q. Blackwell and L. J. Fosdicks, *Am. J. Physiol* **195**, 712 (1958).
8. R. H. Persellen, *Arth. Rheum.* **15**, 144 (1972).
9. A. A. Dietz, H. M. Rubinstein and L. Hodges, *Clin. Chem.* **20**, 396 (1974).
10. P. S. Hench, E. C. Kendell, C. H. Slocumb and H. F. Polley, *Ann. rheum. Dis.* **8**, 97 (1949).
11. P. S. Hench, *Proc. Mayo Clin.* **8**, 430 (1933).
12. R. S. Pinals, *Ann. rheum. Dis.* **32**, 471 (1973).

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The presence of EDTA in commercial preparations of isocitrate dehydrogenase

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We communicate this note merely to call attention to the presence of EDTA in Sigma Type IV isocitrate dehydrogenase preparations (IV-ICD) and certain consequences thereof. In isolated microsomal preparations, iron-stimulated lipid peroxidation results in degradation of phospholipids and is accompanied usually by a reduction in mixed function oxidase activity [1]. EDTA, in micromolar concentrations, inhibits lipid peroxidation and usually enhances microsomal enzymatic activity [2, 3]. In addition, cytosol, upon recombination with the microsomal pellet, inhibits lipid peroxidation and enhances the oxidation of substrates [4].

Most investigations involving the hepatic microsomal mixed function oxidase complex have employed either glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase in the NADPH generating system. The commonly utilized IV-ICD has recently been found to contain a factor, separable from the dehydrogenase-protein by gel filtration, which inhibits microsomal lipid peroxidation and enhances microsomal mixed function oxidase activity [5]. Certain properties of the factor in IV-ICD, such as its apparent molecular weight (10,000 daltons) upon gel filtration, its heat stability, and its effects on lipid peroxidation and mixed function oxidase activity, suggested possible identity with the hepatic cytosol factor currently under investigation in different laboratories [5–7].

Discussion with Sigma Chemical Company, St. Louis, Mos., revealed that IV-ICD is prepared by Method II of the procedure of Siebert *et al.* [8] through and including Step 5 which involves dialysis against 10 mM EDTA. Most investigators utilize an assay system for mixed function oxidase activity that involves approximately a 1:2500 final dilution of IV-ICD. If the original EDTA concentration were 10 mM, final concentrations would approximate 4 μM, a concentration well within the range in which EDTA has been found to influence lipid peroxidation and mixed function oxidase activity.

Spectral data indicate the expected presence of at least 10 mM, and more likely ca. 40, mM EDTA in IV-ICD (Fig. 1). The absorption spectra (600–800 nm) of EDTA cupric ion complex and of mixtures of cupric ion and IV-ICD, before or after boiling and centrifugation to remove denatured protein were identical. The inclusion of glycerol with EDTA, as in IV-ICD, did not alter results. The effects of IV-ICD and EDTA on microsomal ethylmorphine *N*-demethylation and lipid peroxidation are compared in Fig. 2. Upon serial dilution, the maximal effects of IV-ICD and EDTA on both processes were identical, but IV-ICD was about twice as potent as 10 mM EDTA. Dialysis, but not heat denaturation, of IV-ICD resulted in the complete loss of effect on *N*-demethylation, lipid peroxidation, and chelate spectral properties. The gel filtration characteristics of the active factor in IV-ICD preparations may reflect the recently reported anomalous behaviour of EDTA upon Sephadex G-25 gel filtration [12].

These data suggest that the action of IV-ICD on the forementioned aspects of mixed function oxidase activity reflects the presence of EDTA. The higher potency of IV-ICD, as compared to 10 mM EDTA, could indicate an additional mode of action, but the spectral chelate data