Lysosomal enzyme activity in rats with adjuvant-induced arthritis

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Evidence is increasing that lysosomal enzymes play an important role in inflammation (de Duve and Wattiaux, 1966; Weissmann, 1967; Houck, 1968; Fell, 1969; Dingle, 1969). Increased activities have been reported in human rheumatoid synovia and synovial membrane, and in other inflamed tissues (Smith and Hamerman, 1962; Luscombe, 1963; Barland, Novikoff, and Hamerman, 1964; Lehman, Kream, and Brogna, 1964; Kerby and Taylor, 1967; Weissmann, Spilberg, and Krakauer, 1969). Recent work suggests that cells which migrate to inflammatory sites, which include blood polymorphonuclear leucocytes and tissue macrophages, may partially discharge their lysosomal enzymes into the extracellular spaces, probably as a result of excessive endocytosis (Weissmann, 1967; Fell, 1969).

It has been suggested that the acidic anti-inflammatory drugs, phenylbutazone, mefenamic and flufenamic acids, ibufenac, and indomethacin, may exert their beneficial effect by partially inhibiting some of these enzymes (Anderson, 1968, 1969a), or in common with anti-inflammatory steroids and chloroquine (de Duve, Wattiaux, and Wibo, 1963; Weissmann, 1964) by stabilizing cell and lysosome membranes (Tanaka and Iizuka, 1968).

Enzyme activity has also been detected in certain experimental inflammations in animals, including rat paws made oedematous by formalin, serotonin, dextran (Kalbhen, 1963; Domenjoz and Mörsdorf, 1965), or carrageenin injection (Coppi and Bonardi, 1968).

It appeared to be of interest to know whether similar increases in lysosomal enzyme activity occur in rats made polyarthritic by injection of Freund's complete adjuvant. This experimental model of systemic chronic inflammation (Pearson, 1956; Newbould, 1963), some symptoms of which resemble those in human rheumatoid arthritis, is used extensively for detecting and evaluating compounds with anti-inflammatory properties. It is concluded that the extensive tissue breakdown in adjuvant arthritis is due to the release and degradative action of lysosomal enzymes on connective tissue components. Effects on these inflammatory changes after the oral

administration of phenylbutazone, hydrocortisone, and D-penicillamine are described and discussed. A preliminary account of part of this work has already been published (Anderson, 1969b).

Materials and methods

MATERIALS

Human tubercle bacilli (methanol dried) were obtained from the Ministry of Agriculture Laboratories, Weybridge, Surrey, England. Hydrocortisone was supplied by Merck Sharpe and Dohme, Ltd., Hoddesden, Herts., England. Other drugs and chemicals were obtained from sources described elsewhere (Anderson, 1968, 1969a). The foot volume meter was manufactured by Ugo Basile, Milan.

PREPARATION OF ADJUVANT INOCULUM

The inoculum consisted of 25 mg. tubercle bacilli in 5 ml. liquid paraffin. This suspension was homogenized for 15 min. to a smooth consistency in a Potter-Elvehjem homogenizer.

INDUCTION OF ADJUVANT ARTHRITIS

24 female Wistar rats (200–250 g.), maintained on a standard pellet diet, were divided into eight groups containing three rats each. Arthritis was induced in the rats in seven groups by the subcutaneous injection of adjuvant inoculum (0.05 ml.) into the right hind paw. The remaining group of uninjected rats served as the control. At intervals within a 41-day period, the volumes of the right and left hind paws of the three rats in each group were measured. The rats were then killed by ether inhalation, about 5 ml. blood obtained by heart puncture for seromucoid (serum glycoprotein) estimation, and both hind paws amputated below the tibia.

PREPARATION OF PAW EXTRACTS

All operations were performed, where possible, at 4° C. The amputated right and left hind paws were pooled separately, cut into small pieces with scissors and homogenized in 0.05M-tris buffer (pH 8), containing 0.15M-NaCl and 0.1 per cent. (v/v) Triton X-100, using 2 ml. buffer for each paw. The incorporation of Triton X-100 ensured lysis of cell membranes during homogenization and thus the release of lysosomal enzymes into the medium. Three paws were homogenized together. Homogenization was carried out for 3 min. in a liquidizer

fitted with metal blades revolving at 1,700 r.p.m. Fluid was decanted off and retained, and the residue re-homogenized under the same conditions, with the same amount of buffer. The resulting homogenate was centrifuged and the supernatant fluid added to the decanted fluid obtained above. The combined extract was centrifuged, divided into three portions and stored at -20°C . Each portion was estimated for β -glucuronidase, acid phosphatase, or collagenolytic activity, as described below, and the units of enzyme activity present in each paw were calculated. Because of enzyme lability, estimations were not done on portions that had undergone more than one freeze-thaw cycle.

ENZYME ESTIMATIONS

β -Glucuronidase was estimated using phenolphthalein- β -glucuronide (0.1 per cent., w/v, in water) as substrate. To the paw extract (0.1 ml.) was added 0.9 ml. 0.2M-sodium acetate buffer (pH 5.4) followed by 0.1 ml. substrate. After incubation for 1 hr. at 37°C ., 2 ml. glycine solution (prepared as previously described, Anderson, 1968) were added, the mixture was centrifuged, and the liberated phenolphthalein in the supernatant fluid was measured spectrophotometrically at 545 m μ . Results were expressed in β -glucuronidase units per paw, 1 unit being that activity of enzyme required to release 1 mg. phenolphthalein from the substrate, under the assay conditions.

Acid phosphatase was estimated using β -glycerophosphate as substrate. This was prepared as previously described (Anderson, 1968), except that sucrose was omitted. To the paw extract (0.1 ml.) was added 2 ml. 0.2M-sodium acetate buffer (pH 5) followed by 0.3 ml. substrate. To allow for phosphate initially present in the paw extract, a control tube contained the same ingredients except that 0.3 ml. buffer was added in place of substrate. To allow for free phosphate in the substrate, a second control tube contained the same ingredients except that 0.1 ml. 0.9 per cent. (w/v) NaCl was added in place of paw extract. After incubation for 1 hr. at 37°C ., the liberated phosphate was measured by the method of Gianetto and de Duve (1955). Results were expressed in acid phosphatase units per paw, 1 unit being that activity of enzyme required to release 1 mg. phosphate (as P) from the substrate, under the assay conditions.

The collagenolytic enzyme was estimated following the method of Anderson (1969a). To 1 ml. paw extract was added 2 ml. 0.2 M-sodium acetate buffer (pH 3.45) followed by 0.5 ml. suspended insoluble collagen (10 mg.). To allow for soluble collagen already present in the paw extract, a control tube contained the same ingredients except that 0.5 ml. 0.9 per cent. (w/v) NaCl was added in place of collagen suspension. After incubation for 3 hrs at 37°C ., the amount of collagen 'solubilized' by the collagenolytic enzyme was measured as previously described (Anderson, 1969a). Results were expressed in collagenolytic enzyme units per paw, 1 unit being that activity of enzyme required to liberate 1 μg . soluble collagen (as hydroxyproline) from insoluble collagen, under the assay conditions.

SEROMUCOID ESTIMATION

Seromucoid (serum glycoprotein, mucoprotein) was esti-

mated as described elsewhere (Lockey, Anderson, and MacLagan, 1956) with reconstituted freeze-dried human serum (Glaxo Laboratories Ltd., Greenford, Middlesex, England) of known protein content as the standard. Results were expressed as mg. seromucoid (estimated as protein) per 100 ml. serum.

FORMULATION OF DRUGS FOR ORAL FEEDING

An appropriate amount of phenylbutazone or hydrocortisone was ground in a mortar with 0.25 ml. Tween-80 and 4.75 ml. carboxymethylcellulose (0.5 per cent., w/v, in water), followed by homogenization to a smooth consistency in a Potter-Elvehjem homogeniser. A portion (0.5 ml.) of the resulting suspension was fed orally to the rat. D-penicillamine (base) was fed to the rats as a solution in 0.5 ml. 0.9 per cent. (w/v) NaCl.

ORAL FEEDING OF DRUGS TO ADJUVANT ARTHRITIC RATS

Sixteen rats, divided into four groups containing four rats each, were made arthritic by injection of adjuvant inoculum. One group received no further treatment and served as a control. The rats in the other three groups received ten daily oral doses of phenylbutazone, hydrocortisone, or D-penicillamine, at levels of 100, 20, and 400 mg./kg. respectively, beginning 5 days after the injection of adjuvant. Paw volumes were measured at intervals. The rats were killed 15 days after inoculation, paw extracts prepared, and lysosomal enzymes assayed.

HISTOLOGICAL METHODS

Tissue samples were rapidly frozen by immersion in isopentane cooled to -70°C . with solid CO_2 . Sections 10 μ thick were cut on a cryostat, mounted on cover slips, air dried for 10 min., and stored at -20°C . Sections were stained with haematoxylin and eosin, and for β -glucuronidase (Hayashi, Nakajima, and Fishman, 1964) and acid phosphatase (Burstone, 1958) activities.

Results

The course of inflammation after adjuvant inoculation, as measured by volume increases of the injected right hind paw, is shown in Fig. 1. Also

FIG. 1 Increase in volume of hind paws of rats after inoculation of adjuvant into right hind paw.

shown are volume increases of the uninjected left hind paw, following the development of secondary lesions. Figs 2 to 4 show increases in β -glucuronidase, acid phosphatase, and collagenolytic enzyme activities, respectively, in the paw extracts. These rises paralleled paw volume increases. Serumucoid levels, which rose steadily throughout the 41 day period as shown in Fig. 5, paralleled increases in paw volumes and enzyme activities. The relationship between serumucoid and acid phosphatase is shown in Fig. 6.

Compared with normal rats, histological examination of paws from rats with adjuvant arthritis

FIG. 2 β -Glucuronidase activity in paws of rats with adjuvant arthritis.

FIG. 3 Acid phosphatase activity in paws of rats with adjuvant arthritis.

FIG. 4 Collagenolytic enzyme activity in paws of rats with adjuvant arthritis.

FIG. 5 Serumucoid (serum glycoprotein, mucoprotein) levels in rats with adjuvant arthritis.

FIG. 6 Relationship between serumucoid (serum glycoprotein) levels and acid phosphatase activity in paws of rats with adjuvant arthritis.

showed pronounced increases in certain cells, mainly polymorphonuclear leucocytes and macrophages, together with increased β -glucuronidase and acid phosphatase activities.

The effect on the polyarthritis of daily oral doses of phenylbutazone, hydrocortisone, and D-penicillamine is shown in Fig. 7. Phenylbutazone, and to a lesser extent hydrocortisone, arrested inflammation of the injected right paw, and greatly retarded the appearance of 'secondary' lesions in the uninjected

acid phosphatase, and collagenolytic enzyme activities in paw homogenates obtained from normal and arthritic rats, and from arthritic rats after oral feeding or drugs. Activities of the three enzymes in the paws of arthritic rats after treatment with phenylbutazone and hydrocortisone were appreciably lower than the levels in the untreated arthritic rats. Penicillamine treatment, however, caused only slight reductions in oedema and enzyme activities. These results were the average of two experiments.

Discussion

The present study has shown that the increase in oedema of both hind paws after adjuvant injection into the right hind paw (Fig. 1) is paralleled by increases in the activities of β -glucuronidase, acid phosphatase, and a collagenolytic enzyme in paw homogenates (Figs 2 to 4). The role of acid phosphatase in inflammation is unknown, but it is probable that β -glucuronidase, in conjunction with lysosomal hyaluronidase, degrades synovial hyaluronic acid and the chondroitin sulphate moiety of cartilage chondromucoprotein (Anderson, 1968), thus contributing to joint inflammation.

Less is known of mammalian enzymes which degrade collagen, and their effect on the inflammatory process. They have been detected in cirrhotic liver and subcutaneous granuloma tissue (Bazin and Delaunay, 1966), in rheumatoid synovial membrane (Harris and Krane, 1969), and in other tissues as discussed elsewhere (Anderson, 1969a). A collagenolytic enzyme which 'solubilizes' insoluble collagen, and which appears to be a form of cathepsin D with optimum activity at pH 3.45, is present in a rat liver lysosome-rich fraction (Anderson, 1968). Rat leucocytes, which were more numerous in the paws of adjuvant arthritic rats than in normal paws,

FIG. 7 Paw oedema in rats receiving oral phenylbutazone (●), hydrocortisone (□), and D-penicillamine (△) compared with untreated rats (○). The drugs were fed daily on Days 5 to 15 after adjuvant inoculation.

left paw. Penicillamine had less effect on both the 'primary' and 'secondary' lesions.

The Table shows the levels of β -glucuronidase,

Table Comparison of lysosomal enzyme activities in paw extracts obtained from normal rats, and from adjuvant-arthritic rats with and without drug treatment

Enzyme activity			Acid phosphatase (units)		β -glucuronidase (units)		Collagenolytic enzyme (units)	
Paw extract			Left	Right	Left	Right	Left	Right
Rats	Normal		0.15		0.83		71	
	Arthritic	Untreated	0.61	2.35	1.29	6.89	133	187
		Fed phenylbutazone (100 mg./kg.)	0.27	1.14	0.93	5.15	77	139
		Fed hydrocortisone (20 mg./kg.)	0.24	0.99	0.92	3.70	56	96
		Fed D-penicillamine (400 mg./kg.)	0.63	1.58	1.07	5.03	116	171

contain a similar enzyme with the same optimum pH value, and two other enzymes which 'solubilize' insoluble collagen, a minor and major one optimally active at pH values 6.2 and 8.4, respectively (A. J. Anderson, unpublished results). The two latter enzymes may partially degrade insoluble collagen extracellularly, the degradation products being taken into cells by endocytosis and degraded further in phagolysosomes by the former enzyme. Collagen breakdown by a two stage process such as this has been suggested by Fell (1969).

The increase in seromucoid levels in the course of adjuvant arthritis, first observed by Weimer, Wood, and Pearson (1968), was confirmed in this study (Fig. 2). Levels of this non-specific acute phase reactant paralleled increases in paw oedema and lysosomal enzyme activity in paw homogenates (Fig. 6). There is a similar relationship between seromucoid levels and inflammation in humans (Lockey and others, 1956). The significance of the increased seromucoid fraction and, indeed, the functions of its constituent glycoproteins are unknown. It has been suggested (Winzler, 1964) that humoral factors released from inflamed sites or from infiltrated leucocytes (Darcy, 1968) may stimulate the liver to increase seromucoid synthesis.

The observed correlation between paw oedema and lysosomal enzyme activity, in both the injected and the uninjected hind paws, suggests increased vascular permeability associated with the accumulation of leucocytes in connective tissue, the subsequent oedema becoming established as blood proteins escape. It is probable that leucocytes liberate lysosomal enzymes as a consequence of intense endocytosis (Weissmann, 1967).

There is evidence that appearance of the widespread secondary lesions in this disease, including the development of inflammation in the uninjected left hind paw, may be a response to immunological mechanisms, including autoimmune phenomena and a delayed hypersensitivity reaction against antigens in the tubercle bacilli (Pearson, Wood, and Tanaka, 1968; Tanaka, Tanaka, and Sugiyama, 1968; Jollès, 1969). However, whether these or other mechanisms yet unknown are responsible for the initiating process, the inflammatory lesions, which in the left hind paw develop 8 to 10 days after adjuvant injection, seem to result from connective tissue degradation by lysosomal enzymes. The activity of these enzymes in both primary and secondary lesions may therefore constitute the 'common final pathway' of inflammation, as originally postulated by Weissmann (1966) with reference to human arthritis. However, it is possible that non-lysosomal enzymes, for instance plasmin, may contribute to connective tissue degradation (Lack, Anderson, and Ali, 1961). Plasminogen could be activated by lysosomal

enzymes, as shown for liver lysosomal cytochrome by Ali and Evans (1968). The reduction in paw oedema and in lysosomal enzyme activity in paw homogenates after daily oral feeding of phenylbutazone and hydrocortisone suggests that these drugs can inhibit lysosomal enzyme activity *in vivo*, as phenylbutazone does *in vitro*. Alternatively they may stabilize cell and lysosome membranes, preventing enzyme release. It was found that phenylbutazone at 0.5 mM partially inhibited β -glucuronidase activity in rat paw homogenates and rat leucocytes, in addition to β -glucuronidase activity in rat liver lysosomes (Anderson, 1968).

Although there are claims that D-penicillamine may be an effective drug in human rheumatoid arthritis (Zuckner, Ramsey, Dorner, and Gantner, 1967), it had little effect on the oedema and enzyme activity of inflamed paws of arthritic rats. Of the lysosomal enzymes examined none was inhibited by penicillamine (Anderson, 1968), suggesting that any anti-inflammatory effects it may have must be due to some other property.

This study suggests that one point of biochemical similarity between rat adjuvant arthritis and human rheumatoid arthritis is the extravascular accumulation of lysosome-containing cells, followed by lysosomal enzyme release and the production of lesions by subsequent connective tissue degradation. An increased understanding of these biochemical events in adjuvant-induced arthritis in rats may assist in the evaluation of potential anti-inflammatory drugs in humans and in the elucidation of their mode of action.

Summary

Increased lysosomal enzyme activity in homogenates of adjuvant-injected hind paws, amputated at various time intervals from rats with adjuvant arthritis, paralleled increases in paw volume (oedema). The enzymes investigated were β -glucuronidase, acid phosphatase, and a collagenolytic enzyme capable of 'solubilizing' insoluble collagen. Enzyme activity in non-injected hind paws remained low until they developed secondary lesions. Histological examination of inflamed paws showed extensive leucocyte infiltration. Oral administration of phenylbutazone and hydrocortisone to arthritic rats arrested increases in both paw oedema and enzyme activity. D-penicillamine had less effect. It is postulated that, in adjuvant-induced arthritis, increased vascular permeability causes an accumulation of leucocytes in connective tissue, followed by liberation of their lysosomal enzymes, while escaped blood proteins stabilize oedema. The beneficial action of anti-inflammatory drugs may result from lysosomal

enzyme inhibition, stabilization of cell or lysosome membranes, or decreased influx of leucocytes.

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RÉSUMÉ

L'activité de l'enzyme des lysosomes chez les rats atteints d'arthrite provoquée par l'adjuvant

L'activité augmentée de l'enzyme des lysosomes des homogénates des pattes arrières injectées d'adjuvant, et amputées à des intervalles variés montraient des augmentations parallèles dans le volume des pattes (oédème). Les enzymes étudiés étaient le β -glucuronidase, l'acide phosphatase, et un enzyme collagénolytique capable de rendre soluble le collagène insoluble. L'activité de l'enzyme dans les pattes arrières non-injectées restait basse jusqu'au développement de lésions secondaires. L'examen histologique des pattes enflammées montrait une infiltration leucocytaire étendue. L'administration buccale de phénylbutazone et d'hydrocortisone aux rats arthritiques avait arrêté les augmentations de l'oédème de la patte ainsi que l'activité de l'enzyme. La pénicillamine-D avait moins d'effet. Il est postulé que dans l'arthrite provoquée par l'adjuvant, une augmentation de la perméabilité vasculaire cause une accumulation de leucocytes dans le tissu conjonctif, suivie de la libération des enzymes des lysosomes, tandis que les protéides sanguins qui avaient échappé stabilisent l'oédème. L'action salutaire des médicaments anti-inflammatoires peut résulter de l'inhibition causée par l'enzyme des lysosomes, la stabilisation de la membrane de la cellule ou celle des lysosomes, ou l'arrivée diminuée des leucocytes.

SUMARIO

Actividad de la enzima lisosomal en ratas con artritis inducida por adyuvante

La incrementada actividad enzimática lisosomal en homogenatos de patas traseras inyectadas con adyuvante, amputadas a diferentes intervalos, de ratas con artritis adyuvante, era similar a los aumentos en el volumen de las patas (edema). Las enzimas estudiadas fueron glucuronidasa β , fosfatasa ácida y una enzima colagenolítica capaz de 'solubilizar' el colágeno insoluble. En las patas traseras no inyectadas, la actividad enzimática fue baja hasta que desarrollaron lesiones secundarias. El examen histológico de las patas inflamadas reveló extensa infiltración de leucocitos. La administración oral de fenilbutazona e hidrocortisona a ratas artríticas impidió aumento tanto del edema de la pata como de la actividad enzimática. La penicilamina D produjo menos efecto. Se supone que, en la artritis inducida por adyuvante, la mayor permeabilidad vascular causa una acumulación de leucocitos en el tejido conectivo, seguida de liberación de sus enzimas lisosomales, en tanto que las proteínas que escapan de la sangre estabilizan el edema. La acción benéfica de las drogas antiinflamatorias quizá se derive de la inhibición de la enzima lisosomal, la estabilización de células o membranas lisosomales, o la reducción del influjo de leucocitos.