Effect of Adjuvant Arthritis on Collagenase and Certain Lysosomal Enzymes in Relation to the Catabolism of Collagen

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Abstract

The activity of collagenase, cathepsin B1, cathepsin D and hyaluronidase was determined in skin, bone, liver, kidney, spleen and serum of adjuvant induced arthritic rats during the acute and chronic phase of the disease. Collagenase was assayed directly in tissue extract by a solution method using radioactive labelled substrate. The activity of collagenase, cathepsin B1 and D was found to increase significantly at both phases of the disease. The activity of hyaluronidase decreased significantly in liver, kidney and spleen of arthritic rats, while in skin, bone and serum no significant change was observed. The results are discussed with respect to catabolism of collagen in adjuvant induced arthritis.

Prednisolone and L-thyroxine were administered to arthritic rats and the activity of collagenase, cathepsin B1, cathepsin D and hyaluronidase was determined in the treated groups during the acute and chronic phase of the disease. Prednisolone was found to suppress the development of arthritis which, in turn, decreased the increased activity of collagenase and lysosomal enzymes cathepsin B1 and D in tissues and serum of arthritic rats. L-Thyroxine was found to slowly diminish the development of inflammation and its beneficial action was found in mesenchymal tissues and skin of arthritic rats but not in bone.

Introduction

Adjuvant induced arthritis is an experimental disease produced by an injection of Freund's adjuvant and serves as a model for human rheumatoid arthritis [1-5]. Adjuvant induced arthritis in rats reflects articular changes and lesions in various parenchymal tissues during the acute and chronic phase of the disease [6-10]. These articular changes and lesion in adjuvant induced arthritis are accompanied by an increase in the activity of certain lysosomal enzymes like acid phosphatase, β-glucuronidase, β-galactosidase, arylsulphatase, β-aspartyl glycosamino hydrolase, α-mannosidase, N-acetyl-β-D-glucosaminidase and collagenolytic enzymes capable of solubilizing collagen in tissues [11-16]. Among the connective tissue proteins, collagen is most affected by this chronic inflammatory disease. TRNAVSKA et al. [17] found an increase in the breakdown of newly formed collagen in the acute phase and a retardation in the conversion of soluble to insoluble collagen in the chronic phase of arthritis. A decreased synthesis of collagen accompanied by an increased catabolism and an impairment of collagen cross-linking during the chronic phase of this inflammatory disease were reported recently [18, 19]. A decrease in lysosomal stability usually increases the level of lysosomal enzymes in extracellular fluid [20-22] which in turn may help the further degradation of collagen after collagenolysis [13, 23]. In this communication, the activity of collagenase and certain lysosomal enzymes which are involved in the catabolism of collagen, namely, cathepsin B1, cathepsin D and hyaluronidase, was determined in various tissues and serum of adjuvant induced arthritic rats. As the administration of prednisolone and L-thyroxine to adjuvant induced arthritic rats was found to improve the changes both in metabolism and cross-linking of collagen [24, 25], the effects of prednisolone and L-thyroxine on the activity of these enzymes were also studied in arthritic tissues and the results are discussed with respect to the catabolism of collagen.

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**Materials and methods**

**Adjuvant arthritis**

Male albino rats initially weighing about 60 g each were used. The adjuvant group received 0.1 ml Freund’s adjuvant per animal injected intradermally into the dorsum of the tail root. The adjuvant contained 10 mg heat-killed *Mycobacterium tuberculosis* (T.B. Chemotherapy Centre, Madras) per ml sterile paraffin oil. Both the arthritic and control animals received commercial diet supplemented with required vitamins and tap water *ad libitum*. The progress of the disease was evaluated by scoring arthritic index and measuring the hind paw volume. Only animals with fully developed polyarthritis were selected for the analysis. The arthritic group was further divided into three groups, namely, Arthritic (A), Arthritic + Prednisolone (AP) and Arthritic + L-Thyroxine (AT). After the development of arthritis, from about the 14th day after the injection of Freund’s adjuvant, daily injections of prednisolone (5 mg/kg) and L-thyroxine (250 μg/kg) were given intra-subcutaneously to the rats in the AP group and AT group respectively. The protective action of prednisolone and L-thyroxine was evaluated by comparing the arthritic score and index with the arthritic group. Body weights were recorded at weekly intervals. Rats were killed by decapitation and the neutral-salt soluble collagen in injected intraperitoneally to each. After 6 h, the animals were used and 200 gCi of 14C-glycine (BARC, Bombay) was thawed three times and centrifuged at 15,000 x g for 60 min. The supernatant was dialysed against several changes of the ice-cold 0.5 M Tris-HCl buffer containing 0.2 M NaCl, containing 0.5–1.5 mg protein per ml was used for collagenase. The supernatant was dialysed against several changes of the same Tris-HCl buffer to produce a final urea concentration of 0.005 M. The enzyme extract was prepared by homogenizing the weighed bone, liver, kidney and spleen were removed immediately and stored at −20°C. The enzyme extract was prepared by homogenizing the weighed tissue samples separately in a pestle mortar or Potter Elvehjem type homogenizer using ice-cold 0.2 M sucrose without any adhering tissues and cut into small pieces with a bone cutter. Liver, kidney and spleen were used as such. The enzyme extract was prepared by homogenizing the weighed tissue samples separately in a pestle mortar or Potter Elvehjem type homogenizer using ice-cold 0.2 M sucrose and diluted to a final concentration of 10%. The resulting homogenate was allowed to stand in ice for 60 to 75 min and then centrifuged at 15,000 × g for 30 min. The supernatant was used as the source of the enzyme for the assay of cathepsin B1, cathepsin D and hyaluronidase.

For collagenase, the weighed tissue samples were homogenized separately in a Teflon glass homogenizer using ice-cold 0.5 M Tris-HCl buffer containing 0.2 M NaCl, 0.005 M CaCl2 and 5 M urea, the extract was frozen and thawed three times and centrifuged at 15,000 × g for 60 min. The supernatant was dialysed against several changes of the same Tris-HCl buffer to produce a final urea concentration of 0.01 M and stored at −20°C. The supernatant containing 0.5–1.5 mg protein per ml was used for collagenase assay.

**Preparation of substrate for collagenase**

30-day-old guinea-pigs, weighing 250–300 g, were used and 200 μCi of 14C-glycine (BARC, Bombay) was injected intraperitoneally to each. After 6 h, the animals were killed by decapitation and the neutral-salt soluble collagen was prepared from the skins and purified by the method of LEVENE and GROSS [26]. The purified sample, after dialysis with 0.01 M acetic acid, was lyophilized. The specific radioactivity of lyophilized collagen was found to be 2500 c.p.m./mg.

**Collagenase assay**

The activity of collagenase was assayed as described by TERATO et al. [27]. In brief, the reaction mixture containing 100 μl of 0.4% (w/v) 14C-labelled collagen dissolved in 0.005 M acetic acid, 100 μl of 0.1 M Tris-HCl buffer, pH 7.8, containing 0.4 M NaCl, 10 mM CaCl2, and 1 M glucose and 200 μl of enzyme preparation was incubated for 4 h at 35°C (a temperature between the denaturation temperatures of native collagen and its enzyme digest). The enzymic reactions was stopped by adding 20 μl of 80 mM o-phenanthroline dissolved in 50% dioxane and keeping the mixture at the same temperature for 60 min and then at room temperature. The reaction products thus denatured were extracted by vigorous shaking after addition of 0.4 ml of dioxane and centrifuged at 6000 r.p.m. for 20 min to precipitate residual undigested collagen. 0.5 ml of the supernatant was mixed with 10 ml of Bray’s solution and assayed for 14C-radioactivity in an Automatic Liquid Scintillation System LSS 34 (ECIL, Hyderabad). To eliminate the interference by denatured collagen, triplicate assays (Water blank, Trypsin blank) were performed. Trypsin (2 × crystallined) was added at a concentration of 25 μg/ml of incubation medium. After subtracting water and trypsin blank, the results were expressed as c.p.m./mg protein (Table 1).

**Disc electrophoresis**

To verify that the enzyme extracted was a true collagenase, collagen and its degradation products were examined by disc gel electrophoresis. Reactions between collagen and collagenase at 25°C were stopped by the addition of 2 M urea and 1% sodium dodecyl sulphate in 100 mM sodium phosphate buffer, pH 7.2, and the collagen products were denatured by heating at 50°C for 30–60 min. Electrophoresis of the samples was carried out at 6 mA per tube on sodium dodecyl sulphate polyacrylamide gels (5%) for 5 h as described by FURTMAIR and TIMPL [28]. Gels were stained with 0.25% Coomassie Brilliant Blue and destained using 5% methanol/7.5% acetic acid.

**Assay of cathepsin B1**

Cathepsin B1 activity was measured by the method of BARRETT [29], using N-α-benzoyl-DL-arginine-p-nitroanilide-HCl (BAPA) as a substrate. The activity was expressed as μM p-nitroaniline/mg protein/h (Table 2).

**Assay of cathepsin D**

Cathepsin D activity was determined using haemoglobin (2% w/v in sodium formate buffer, pH 3.5, at 45°C for 2 h) and the release of soluble tyrosine was determined in the filtrate by the method of HANLEY et al [30]. The activity was expressed as μg tyrosine liberated/mg protein/h (Table 3).

**Assay of hyaluronidase**

Hyaluronidase activity was assayed after incubation at 37°C with the exogenous substrate hyaluronate (100 μg/250 μl assay mixture). The newly formed terminal N-acetyl glucosamine was measured by the colorimetric