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Leucocyte infiltration and cartilage proteoglycan loss in immune arthritis in the rabbit

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1 The relationship between phagocytic leucocyte infiltration and cartilage degradation in immune arthritis has been investigated in groups of normal and neutropenic rabbits.

2 Injection of antigen into the knee joints of sensitized control animals induced joint swelling, prostaglandin E_2 (PGE_2) synthesis, leucocyte accumulation and proteoglycan loss from articular cartilage.

3 Intravenous injection of nitrogen mustard caused a selective depletion of circulating neutrophils and monocytes with little or no effect on platelets or lymphocytes. In neutropenic animals challenged with antigen, there was virtually no joint swelling, PGE_2 synthesis or leucocyte infiltration but cartilage proteoglycan loss was unchanged after 1 day and increased by day 4 compared to control animals.

4 The numbers of circulating leucocytes returned to normal 3–4 days after nitrogen mustard treatment and leucocyte infiltration occurred in antigen-challenged joints but this was not accompanied by joint swelling. Subsequent intra-articular injection of PGE_2 did, however, cause swelling.

5 Lysosomal enzyme levels in arthritic joint fluids were measured. The levels of β-glucuronidase, which is released by activated phagocytes, were decreased in neutropenic animals but the levels of N-acetyl-β-glucosaminidase, which is a marker of tissue damage, were not changed by neutrophil depletion.

6 Intra-articular injections of the cytokine interleukin-1 (IL-1) induced a pattern of leucocyte infiltration and cartilage proteoglycan loss similar to that seen in immune arthritis. In neutropenic animals, IL-1 did not cause significant accumulation of leucocytes in the joint but the loss of proteoglycan from cartilage was unimpaired.

7 These results indicate that both leucocyte infiltration and prostaglandin synthesis are required for joint swelling but that tissue degradation is mediated by resident cells. It is likely that release of IL-1 by synovial cells stimulates the synthesis and activation of metalloproteinases which initiate the process of tissue degradation.

Introduction

Swelling, pain and stiffness in the articulating joints are the symptoms of chronic arthritis which can be alleviated, to some extent, by non-steroidal anti-inflammatory drugs. However, the progressive degradation of cartilage and bone is not inhibited by most available therapies (Wright & Amos, 1980). Arthritic joints are infiltrated with phagocytic leucocytes and it has been suggested that these cells contribute to the joint destruction as well as the acute symptoms of the disease (Weissman, 1972; Davies & Allison, 1978). Although specific stimuli or cell death can result in the release from neutrophils of enzymes such as elastase or cathepsin G, which can degrade cartilage *in vitro* (Oronsky & Perper, 1975), there is no direct evidence that phagocytic leucocytes mediate the cartilate destruction observed in diseases such as rheumatoid arthritis (RA).

In order to elucidate the acute effects of infiltrating leucocytes in the pathogenesis of immune arthritis, we have investigated the effect of depleting the peripheral polymorphonuclear leucocytes (PMN) and monocytes on the development of antigen-induced arthritis in the rabbit, a lesion which closely resembles RA in man (Dumonde & Glynn, 1962). In addition, we have measured cartilage proteoglycan loss in response to intra-articular injection of IL-1 in both normal and neutropenic rabbits.

Some of these results have been reported to the

British Pharmacological Society (Henderson et al., 1987b).

Methods

Induction of antigen-induced arthritis

Adult male New Zealand White rabbits (weight 2.5–3.0 kg) were immunised intradermally with 4 mg ovalbumin (Sigma, Poole, Dorset) in 1 ml Freund's complete adjuvant (Gibco, Paisley, Scotland). Animals were reimmunised 14 days later in the same way. Five days after the second immunisation, arthritis was induced in one knee joint by the intra-articular injection of 5 mg ovalbumin in 1 ml sterile saline. The contralateral knee joint was injected with sterile saline and served as a within-animal control. Some animals received 1 or 1.75 mg kg^{-1} nitrogen mustard (Mustine; Boots, Nottingham) 3 days prior to antigen challenge to deplete the circulating PMN and monocytes. The numbers of circulating leucocytes and platelets were monitored before and after antigen challenge.

Animals were killed 1, 4 or 17 days after antigen challenge and the joint diameters were measured with calipers. Sterile saline (1 ml) was injected into both knee joints of each animal before the joints were opened and the resultant joint washes collected and taken for total and differential leucocyte counts. After centrifugation of the joint wash (10,000 *g* for 3 min), the cell-free supernatant was stored at −20°C and later assayed for the content of prostaglandin E_2 (PGE_2), sulphated glycosaminoglycans (GAGs) and lysosomal enzymes. The articular cartilage was dissected from the ends of the femurs and the proteoglycan content was determined as previously described (Pettipher et al., 1986).

Intra-articular injection of interleukin-1

The effect of intra-articular injection of human recombinant interleukin-1α (IL-1α, Dainippon, Osaka, Japan) was investigated in normal and leucopenic rabbits. IL-1α (25 ng) was injected through the suprapatellar ligament into the joint space and the contralateral knee joint received an equal volume of vehicle (0.5 ml saline containing 0.2% foetal calf serum; Gibco).

Animals were killed 24 h after a single injection and the joint contents washed out with 1 ml sterile saline. Total and differential counts were performed on the infiltrating leucocytes and the levels of sulphated glycosaminoglycans determined in the cell-free joint wash. The proteoglycan and hydroxyproline content of the articular cartilage from the femoral head was also determined.

Assay of prostaglandin E_2

PGE_2 was assayed in the cell-free unextracted joint washes by specific radioimmunoassay (Salmon, 1978). Rabbit antiserum to PGE_2 was incubated with test samples and tritiated standards. Free radioactivity was removed with dextran-coated charcoal and the residual bound activity was measured in a scintillation spectrometer.

Assay of glycosaminoglycans

The proteoglycan content of cartilage or joint washes was determined by assay of sulphated glycosaminoglycans (GAGs; the hetero-polysaccharide side-chains of proteoglycan) following digestion with papain (Sigma) for 1–2 h at 65°C. GAGs were measured by the 1,9-dimethylmethylene blue binding assay described by Farndale et al. (1986). The proteoglycan content of cartilage was expressed as μg GAG per mg wet weight or dry weight of tissue. In some joint washes, GAGs were assayed before and after incubation with chondroitinase ABC (Sigma; 0.05 unit ml^{-1} for 30 min at 37°C).

Assay of hydroxyproline

In some experiments, the collagen content of cartilage was assessed by measurement of hydroxyproline concentrations in the papain digests. Digests were assayed in a Biotronik (Frankfurt, W. Germany) LC 6001 amino acid analyzer and hydroxyproline content was expressed as μg per mg wet weight of cartilage.

Assay of lysosomal enzymes

β-Glucuronidase: 100 μl of diluted cell-free joint wash was incubated with 100 μl 0.8 mg ml^{-1} phenolphthalein glucuronide in 0.05 M acetate buffer (pH 4.5) for 16 h at 37°C. The reaction was terminated with 100 μl 0.4 M sodium carbonate buffer (pH 10) and the absorbance read at 540 nm with a Titertek Multiskan plate reader (Flow Laboratories, Rickmansworth, Herts).

N-acetyl-β-glucosaminidase: 100 μl of cell-free joint wash fluid was incubated with 100 μl 0.1 M citrate-phosphate buffer (pH 4.5) containing 1.5 mg ml^{-1} *p*-nitrophenyl-N-acetyl-β-glucosaminide for 2 h at 37°C. The reaction was terminated with 100 μl 1 M sodium hydroxide/glycine and the absorbance measured at 405 nm. Enzyme substrates and reagents were obtained from Sigma.

Measurement of plasma exudation in rabbit skin

The leakage of plasma in response to bradykinin was examined in neutropenic and control animals at 24 h

after antigen challenge. Plasma exudation was measured by the detection of extravascular leakage of labelled albumin (Williams, 1979). The backs of rabbits were shaved prior to an intravenous injection of 50 μCi of ^{125}I-labelled human serum albumin (Amersham International plc, Aylesbury, Bucks.) in a 2.5% solution of Evans' blue dye. Intradermal injections of 100 μl of a sterile solution of bradykinin (5 μg ml^{-1}) were made in replicates of five. After 30 min the animals were killed and radioactivity measured in skin sites and plasma.

Histology

Samples of synovial lining were dissected and placed in buffered formalin for subsequent paraffin wax embedding and routine histological sectioning. The sections were stained with haematoxylin and eosin.

Results

Circulating leucocytes

Three days after a single intravenous injection of 1.75 mg kg^{-1} nitrogen mustard in sensitized rabbits the circulating numbers of PMN were reduced from $5.3 \pm 1.6 \times 10^6$ cells ml^{-1} blood (mean $\pm$ s.e.mean, $n = 6$) to $< 10^5$ cells ml^{-1}. The numbers of monocytes were reduced from $6.1 \pm 1.1 \times 10^5$ cells ml^{-1} to $1.1 \pm 0.2 \times 10^5$ cells ml^{-1} at this time. In contrast, the number of circulating platelets was not significantly affected ($385 \pm 119 \times 10^6$ cells ml^{-1} at day 0 compared to $485 \pm 49 \times 10^6$ cells ml^{-1} at day 3). The numbers of circulating lymphocytes were only partially reduced from $5.1 \pm 0.6 \times 10^6$ cells ml^{-1} to $3.1 \pm 0.3 \times 10^6$ cells ml^{-1}. The depletion of PMN and monocytes was sustained for two days and the period of neutropenia could be prolonged to 4 days by a second injection of nitrogen mustard, 3 days after the first. The reductions in PMN and monocyte numbers were highly significant ($P < 0.001$).

Leucocyte infiltration and joint swelling in antigen-induced arthritis

Antigen challenge of sensitized animals resulted in high leucocyte infiltration into the joint cavity. At 24 h after challenge these comprised 95 ± 2% PMN and 5 ± 1% monocytes. Later, the proportion of mononuclear cells (including lymphocytes) increased and at day 17 there were 28 ± 9% mononuclear cells. However, the predominant leucocyte infiltrating the joint fluid during the course of this lesion was the PMN. Histologically, the synovial tissue demonstrated diffuse PMN infiltration at day 1 after antigen challenge. At day 4 the PMN infiltration was less intense but more mononuclear cell accumulation was apparent. This leucocyte infiltration was accompanied by joint swelling of 5–7 mm which was sustained during the period of the study (Figure 1).

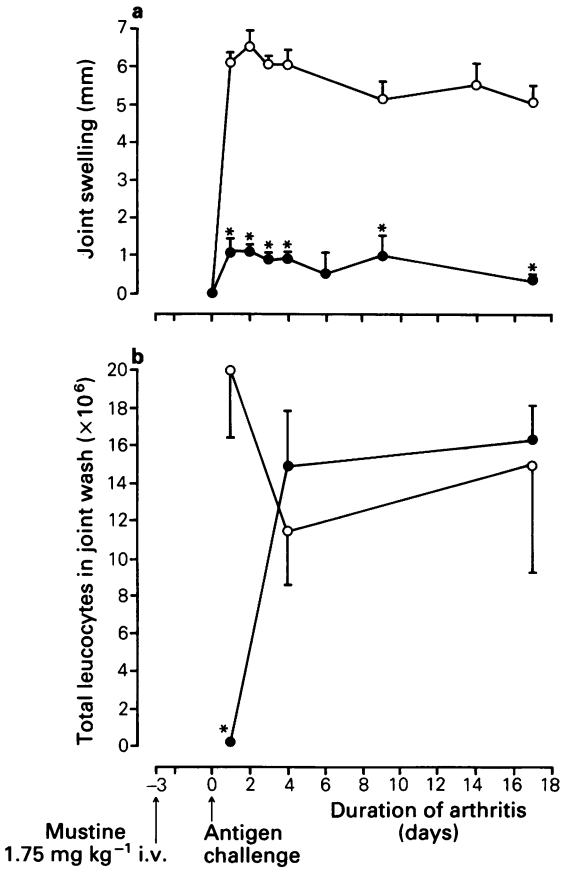

Figure 1 Joint swelling and leucocyte infiltration into arthritic joint fluid in rabbits after the induction of antigen-induced arthritis. Swelling (a) and total leucocyte numbers (b) are shown in control animals (○) and in those previously depleted of circulating PMN and monocytes with a single intravenous injection of nitrogen mustard (●). Each point is the mean from 4–13 animals with s.e.mean shown by vertical bars (* $P < 0.001$ compared to control).

Twenty four hours after antigen challenge in neutropenic rabbits, the numbers of leucocytes in joint fluids were less than 1% of those in control animals. Histologically, the synovial tissue demonstrated a similar lack of leucocyte infiltration at day 1. The absence of leucocytes in the joint was accompanied by an almost complete suppression of joint swelling

(Figure 1). By day 4, the leucocyte numbers in animals treated with a single dose of nitrogen mustard had returned to control levels, both in the circulation and in the synovial tissue and fluid. Joint swelling, however, remained suppressed for up to 17 days (Figure 1).

To investigate whether the reduction in swelling in neutropenic animals was associated with the absence of PMNs or through a direct effect of nitrogen mustard on the vasculature, two experiments were carried out. Firstly, nitrogen mustard was administered 2 h before antigen challenge, instead of 4 days before challenge, and joint swelling was observed for 3 days, a period subsequent to nitrogen mustard injection but prior to PMN depletion. In this period, joint swelling was not significantly different from antigen-induced swelling in the joints of animals not treated with nitrogen mustard (Figure 2a). This experiment demonstrates that reduced swelling is associated with nitrogen mustard-induced neutropenia and not just the presence of nitrogen mustard. Secondly, vascular permeability responses in neutropenic animals were investigated in a tissue remote from the joint at a time when intra-articular injection of antigen failed to provoke a swelling response

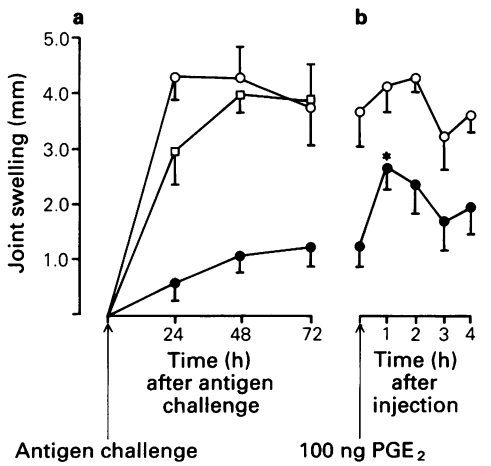

Figure 2 (a) Joint swelling following antigen challenge in normal sensitized rabbits (○) or neutropenic rabbits (●). Neutropenia was induced by a single intravenous injection of nitrogen mustard 3 days before challenge. Swelling was also measured in animals treated with nitrogen mustard just 2 h before challenge (□), a time at which there was no leucocyte depletion. (b) Three days after challenge, the same animals received an injection of prostaglandin E_2 (PGE_2) 100 ng into the antigen-treated joints. This caused a significant increase in joint swelling in the nitrogen mustard-treated animals, but not in the control group. Each point is the mean from 4 animals with s.e.mean shown by vertical bars. (* $P > 0.05$).

in the joint. Intradermal injection of bradykinin (0.5 μg) induced leakage of 30.9 ± 5.3 μl of the plasma marker in a leucopenic animal at day 1 after antigen challenge compared to 26.8 ± 6.7 μl in the control ($n = 5$). This indicates that nitrogen mustard treatment does not impair normal vascular mechanisms in oedema formation, confirming previous observations (Wedmore & Williams, 1981).

Prostaglandin E_2 and joint swelling in antigen-induced arthritis

Approximately 0.7 ml joint wash was collected from each joint and the volume recovered from swollen joints 24 h after antigen challenge (0.74 ± 0.06 ml, $n = 17$) was not significantly different from the volume recovered from the unswollen joints of neutropenic animals (0.67 ± 0.04 ml, $n = 19$). This indicates that most of the joint swelling is accounted for by oedema in the synovial tissues rather than increased synovial fluid volumes. It is possible, however, that there are significant variations in synovial fluid volumes and for this reason, PGE_2, GAG and enzyme levels in joint washes are expressed as total activity recovered per joint.

At day 1 after antigen challenge, arthritic joints contained 8.9 ± 2.4 ng PGE_2 ($n = 6$) whereas in the antigen-injected joints of neutropenic animals there was only 0.5 ± 0.2 ng PGE_2 ($n = 7$). The levels of PGE_2 remained suppressed at day 4 in nitrogen mustard-treated animals even though the level of leucocyte infiltration was not now significantly different from controls.

Intra-articular injection of 100 ng PGE_2 in nitrogen mustard-treated rabbits at day 3, at a time when neutrophils were infiltrating the joint, caused a significant ($P < 0.05$) increase in joint swelling (Figure 2b). Intra-articular injections of PGE_2 did not induce swelling in normal rabbits.

Cartilage proteoglycan loss in antigen-induced arthritis

One day after challenge, the articular cartilage in the antigen-injected joint contained 10–20% less proteoglycan than the cartilage from the contralateral, saline-injected joint. The mean proteoglycan loss, expressed as a percentage reduction in GAGs per mg wet weight of cartilage ($15.5 \pm 2.7\%$, $n = 5$, Figure 3) was not significantly different from the value obtained when proteoglycan loss was expressed as a reduction in GAGs per mg dry weight of cartilage ($13.7 \pm 6.1\%$, $n = 4$). This indicates that reduced proteoglycan content is not due to increased hydration of the tissue and is rather due to net loss of proteoglycan from the cartilage. Proteoglycan loss was not impaired in neutropenic animals (Figure 3).

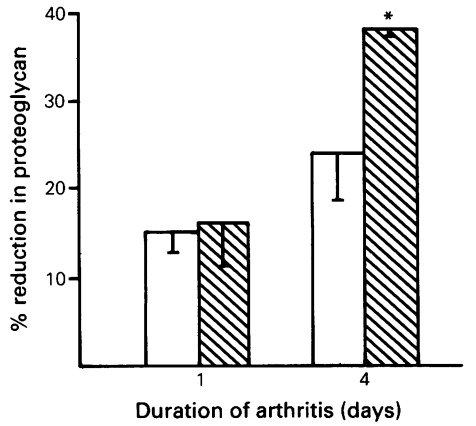

Figure 3 Proteoglycan loss from articular cartilage in arthritic joints from control (open columns) and neutropenic (hatched columns) rabbits. Neutropenia was induced by the intravenous injection of nitrogen mustard (1.75 mg kg^{-1}) 3 days before antigen challenge. After a single injection of nitrogen mustard, neutropenia was only sustained for 2–3 days after challenge. In order to prolong the period of neutropenia, to permit the evaluation of effects on cartilage up to 4 days after challenge, a second injection of nitrogen mustard was given on the day of challenge. Each histogram is the mean of values from 5–9 rabbits with s.e.mean shown by vertical bars. * Indicates a significant difference ($P < 0.05$) between control and neutropenic values.

The reduction in cartilage proteoglycan content was accompanied by an elevation in the proteoglycan content of joint washes. Following papain digestion, joint washes collected 24 h after antigen challenge contained 181 ± 57 μg GAGs ($n = 7$) and this was not significantly different from washes from neutropenic animals which contained 227 ± 63 μg GAGs ($n = 7$). The saline-injected joints of both groups of animals contained less than 20 μg GAGs. Estimates of GAG concentrations in joint washes were reduced by 75–80% following incubation with chondroitinase ABC, indicating that most of the GAG activity is derived from chondroitin sulphate.

In a separate series of experiments, neutropenia in a group of rabbits was prolonged by the intravenous administration of a second dose of nitrogen mustard 3 days after the first. In these animals, leucocyte accumulation in the joints remained suppressed by more than 75% for 4 days after antigen challenge. Cartilage taken from animals not treated with nitrogen mustard showed approximately 23% loss of proteoglycan content 4 days after antigen challenge (Figure 3). This loss was significantly enhanced to almost 40% in neutropenic animals (Figure 3).

Lysosomal enzymes in antigen-induced arthritis

Twenty four hours after antigen challenge, both β-glucuronidase and N-acetyl-β-glucosaminidase activity were elevated in arthritic joint washes, compared to washes from saline-injected joints. However, while the level of β-glucuronidase was reduced in the arthritic washes from neutropenic animals, the level of N-acetyl-β-glucosaminidase remained elevated (Figure 4).

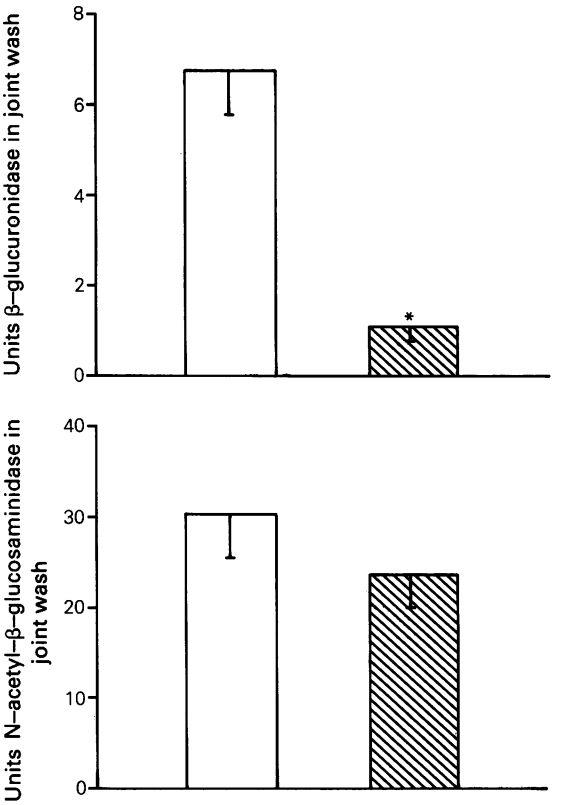

Figure 4 The levels of lysosomal enzymes in the cell-free joint fluid from rabbits with antigen-induced arthritis: (a) shows the level of β-glucuronidase in arthritic joint fluid from control (open columns) and neutropenic (hatched columns) animals. The fluid from control contralateral joints contained <0.5 units activity: 1 unit is that activity which liberates 1 μg phenolphthalein from the substrate per hour at 37°C. In (b) the level of N-acetyl-β-glucosaminidase in arthritic joint fluid from control (open columns) and neutropenic (hatched columns) animals is shown. The fluid from contralateral joints contained <10 units activity: 1 unit is that activity, which liberates 1 μmol of *p*-nitrophenol from the substrate per hour at 37°C. Each point is the mean of values from 6–11 animals with s.e.mean shown by vertical bars (* $P < 0.001$ compared to control).

Effect of interleukin-1 in normal rabbits

Injection of interleukin-1α (25 ng) resulted in high leucocyte infiltration in normal rabbit knee joints. At 24 h the leucocyte composition was 48 ± 7% PMN and 50 ± 9% monocytes ($n = 4$). This effect was accompanied by reduction in the proteoglycan content of the articular cartilage and an elevation of proteoglycan fragments in the joint fluid (Figure 5).

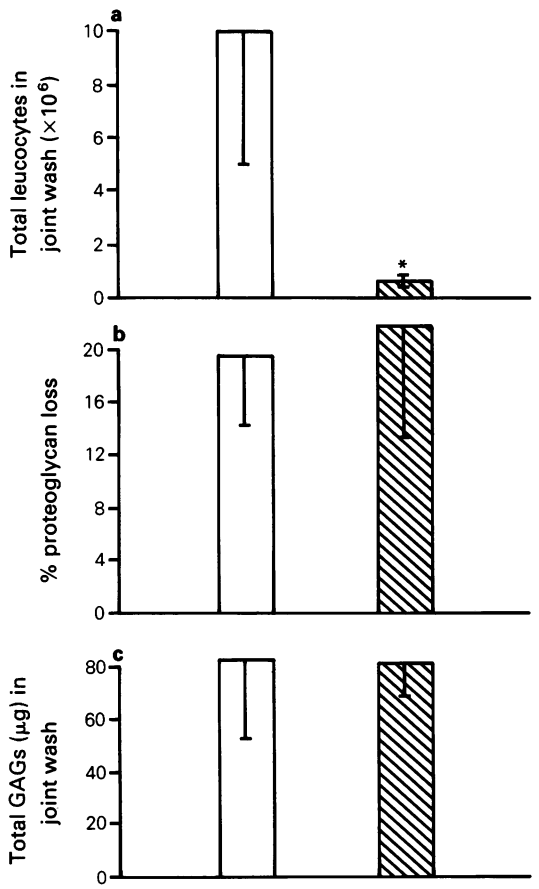

Figure 5 The effect of intra-articular injection of interleukin-1 (recombinant IL-1α; Dainippon) into knee joints of normal (open columns) and neutropenic (hatched columns) rabbits: (a) shows the total leucocyte numbers 24 h after IL-1 injection and (b) shows the percentage reduction in proteoglycan content of the articular cartilage; (c) shows the elevation of glycosaminoglycans (GAGs) in the joint washes of rabbits having received interleukin-1α. Joint washes from contralateral joints contained less than 25 μg glycosaminoglycans. Each column is the mean of values obtained from 4–5 animals and the s.e.mean is shown by vertical bars (* $P < 0.001$ compared with control animals).

Cartilage proteoglycan loss in response to IL-1α was not abrogated in neutropenic rabbits, despite a reduction in the leucocyte accumulation of >95% compared to control animals (Figure 5).

To assess the effect of IL-1 on cartilage collagen content, the hydroxyproline content of papain digests of cartilage taken from IL-1-injected joints were measured. Cartilage from saline-injected joints contained 549 ± 32 ng hydroxyproline per mg wet weight of tissue ($n = 5$) and this was not significantly reduced in cartilage from IL-1-injected joints (481 ± 70 ng). This suggests that proteoglycan is lost from the cartilage prior to collagen loss or degradation. These experiments also indicate that changes in proteoglycan content of the cartilage are not due to increased hydration of the tissue.

Discussion

Within 24 h after induction of antigen-induced arthritis in the rabbit, PMN and monocytes infiltrate the joint, the levels of PGE_2 are elevated in the joint wash and the joint becomes swollen (Pettipher *et al.*, 1986; Henderson & Higgs, 1987a). We have shown that prior depletion of circulating PMN and monocytes with nitrogen mustard abolished the leucocyte accumulation, almost completely suppressed the joint swelling and inhibited the rise in PGE_2 levels. When the rabbits had recovered from the effect of the alkylating agent, at day 3 in the arthritis, PMN and monocytes infiltrated the joint. However, the level of PGE_2 remained low and the joints only became swollen when exogenous PGE_2 was injected intra-articularly. This suggests that although the presence of PMN and monocytes is necessary to elicit the acute symptoms of inflammation in antigen-challenged joints, leucocyte infiltration alone is not sufficient to induce swelling; the concomitant elevation of factors such as PGE_2 is also necessary.

Cartilage degradation is a common finding both in rheumatoid joints and in those of rabbits with antigen-induced arthritis. Proteoglycan loss from articular cartilage is detectable as early as 24 h after antigen challenge and this progresses to 60% loss after 42 days (Pettipher & Henderson, 1988). It is unlikely that neutrophils or monocytes mediate cartilage degradation, for nitrogen mustard treatment inhibited their accumulation in the joint without affecting the loss of proteoglycan from the cartilage or the elevation of proteoglycan in the joint wash observed at day 1 after antigen challenge. This may explain results obtained by other workers who showed that increased recruitment of leucocytes to an inflamed air-pouch did not result in enhanced proteoglycan loss from fragments of implanted cartilage (Sedgwick *et al.*, 1985).

We have previously shown that a single injection of interleukin-1 induces leucocyte infiltration and cartilage proteoglycan loss (Pettipher *et al.*, 1986) and the present study indicates that the cartilage degradation in response to IL-1 is not dependent on the presence of PMN or monocytes. This further dissociates joint damage from leucocyte accumulation and suggests that activation of the resident joint tissues by cytokines, such as IL-1, is the important factor in cartilage destruction. This view is supported by the finding that the elevation of the enzyme N-acetyl-β-glucosaminidase in arthritic joints was not reduced in neutropenic animals. This may be particularly relevant because the synovial tissue and fluid content of this enzyme has previously been reported to correlate with joint damage in RA (Muirden, 1972). Furthermore, activation of the resident synovial lining cell population has been demonstrated both in RA (Henderson & Glynn, 1981) and antigen-induced arthritis (Henderson, 1981). Chondrocytes show a similar activation (Henderson, 1984).

In conclusion, we have shown that the inflammatory components of immune arthritis such as swelling, PGE_2 production and more importantly, leucocyte infiltration, are dissociated from degradative processes, such as cartilage proteoglycan loss. It appears that both PMN accumulation and PGE_2 production are required for acute swelling, but that cartilage degradation occurs independently of these phenomena. It is likely that cytokines, such as IL-1, are mediating this degradation by stimulating directly the synovial cells and/or chondrocytes to release degradative enzymes as is known to occur *in vitro* (Murphy & Reynolds, 1985). The source of IL-1 in arthritic joints may be cells other than monocytes, such as endothelial cells (Stern *et al.*, 1985; Miossec *et al.*, 1986) or synovial cells (Goto *et al.*, 1987). Furthermore, catabolic activity has already been shown to be produced from non-inflamed synovial tissue (Fell & Jubb, 1977) and this activity is likely to be related to IL-1 (Saklatvala *et al.*, 1984).

Interestingly, by day 4 after antigen challenge proteoglycan loss was enhanced in neutropenic animals. This suggests that leucocyte migration may suppress cartilage proteoglycan loss. It is possible, therefore, that the inflammatory response is a protective mechanism against tissue destruction in erosive arthritis. This may have implications both for therapies in use and those proposed for the treatment of chronic inflammatory arthropathies.

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