Biochemical Pharmacology, Vol. 37, No. 21, pp. 4171–4176, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

COMPARISON OF THE *IN VIVO* INFLAMMATORY ACTIVITIES AFTER INTRA-ARTICULAR INJECTION OF NATURAL AND RECOMBINANT IL-1α AND IL-1β IN THE RABBIT

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(*Received* 10 *March* 1988; *accepted* 27 *May* 1988)

Abstract—Comparison has been made of the *in vivo* pro-inflammatory activities or porcine natural and human recombinant α and β interleukin 1 (IL-1) after injection into the knee joints of rabbits. Both forms of pig IL-1 and human IL-1 were separately equiactive *in vitro* in stimulating rabbit synovial fibroblasts and articular chondrocytes to synthesize prostaglandin E_2 (PGE_2). Injection of IL-1 into the rabbit knee joint was not associated with swelling of the joint nor with the appearance of PGE_2 in the synovial fluid. However, all preparations of IL-1 induced a dose-dependent increase in inflammatory leukocytes in the synovial lining and joint cavity. In addition, both the α and β forms of IL-1 from both species caused loss of proteoglycan from the matrix of articular cartilage. This study demonstrates that both genetically distinct forms of IL-1 have the same range of inflammatory actions within the joint and that they have similar potencies in these respects.

Rheumatoid arthritis (RA) is a chronic inflammatory disease primarily affecting the synovial joints. Pain and swelling are the major symptoms in afflicted joints and, in addition, there is progressive destruction of the extracellular matrices of articular cartilage and subchondral bone [1, 2]. The symptoms of RA can be moderated by non-steroidal anti-inflammatory drugs (NSAID). This suggests that products of cyclo-oxygenase activity are largely responsible for these symptoms. However, there is no evidence that NSAID have any effect on the progression of tissue damage [2].

During the past decade it has become established that the cytokine, interleukin 1 (IL-1) stimulates isolated cells derived from joint tissues to secrete eicosanoids and neutral proteinases [2]. Analysis of the synovial fluids from patients with chronic arthritis has revealed the presence of IL-1 [3]. These findings have led to the belief that IL-1 is a key mediator of the pathology of RA [1]. We have recently provided direct evidence to support this hypothesis by demonstrating that intra-articular injection of natural human highly purified IL-1 into the knee joints of rabbits caused the infiltration of inflammatory leukocytes into the joint and the loss of proteoglycan from the matrix of articular cartilage [4].

Using the techniques of gene cloning and expression, it has been established that monocytes contain two genes coding for IL-1. Human monocytes contain complementary DNAs encoding for active forms of IL-1 with an acidic pI of 5 (termed IL-1α) and a molecule of pI 7 (IL-1β) [5]. Similarly, the genes encoding for IL-1α and IL-1β in the mouse have been isolated [6]. In macrophages from both species IL-1β is the major form produced by activated cells [5, 6]. Porcine mononuclear cells have also been reported to secrete two forms of IL-1. These have been purified to homogeneity and have pI values of 5 (IL-1α) and 8.3 (IL-1β) [7].

The α and β forms of IL-1 have been reported to have different potencies in some *in vitro* assays. For example the pI 8 form of pig IL-1 is only a weak stimulator of thymocytes but is equiactive with the pI5 form in other *in vitro* assays [7]. With highly purified human IL-1 differences in potency have also been reported [8]. Human recombinant IL-1α (hr IL-1α) is 40–60-fold more active than hrIL-1β in stimulating resorption of rat long bones [9]. These findings are of interest in light of the reports that both forms of human [10] and pig [11] IL-1 bind to the same receptor.

It has not yet been established what is the predominant form of IL-1 produced in the rheumatoid joint. The different potencies of IL-1α and IL-1β in *in vitro* assays may reflect their actions *in vivo*. To directly test the potencies of the two IL-1s *in vivo* these molecules from pig and human mononuclear cells have been separately injected into the rabbit knee and the inflammatory changes monitored. In addition, the ability of these various preparations of IL-1 to stimulate isolated rabbit cells has also been assessed.

MATERIALS AND METHODS

Source of IL-1. Human highly purified IL-1 prepared from stimulated monocyte cultures was obtained from Genzyme (Suffolk, U.K.). Human Recombinant IL-1 (hr-IL-1) α and β were also obtained from Genzyme. A second source of recombinant molecules was the National Institute of Biological Standards and Control (NIBSC), Hampstead, U.K., which supplied aliquots of freeze-dried IL-1α and IL-1β as part of an international standardization

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protocol. This material came originally from Immunex and has been designated NIBSC IL-1 in this paper. Porcine α and β IL-1 were generously provided by Dr. J. Saklatvala, Strangeways Laboratory, Cambridge, U.K.

Lymphocyte activation factor (LAF) assay. The ability of the various preparations of IL-1 to stimulate murine thymocyte mitogenesis was tested as described [4]. One unit is defined as the amount of IL-1 required to double the proliferative response of mouse thymocytes stimulated with 1 μg ml^{-1} of phytohemagglutinin alone [12].

Preparation of rabbit chondrocytes and fibroblasts. To prepare chondrocytes articular cartilage was dissected from the knee and shoulder joints of young (< 1 kg) New Zealand white rabbits using a method based on that described by Plaas *et al.* [13]. Briefly, cartilage slices were digested for 4 hr at 37° in DMEM (Flow, Paisley, Scotland) containing 0.5% BSA (Sigma, Dorset, U.K.), 500 U/ml penicillin, 500 μg/ml streptomycin (Flow) to which collagenase (type II: Sigma), hyaluronidase (Sigma) and protease (Sigma) had been added. Cells were then washed three times in calcium-free PBS (Wellcome, Kent, U.K.), filtered through a Nitex filter (Cadish, London) to remove undigested cartilage and were aliquoted into 24 well plates (Flow) at a density of 1×10^5 cells/well. Cells were cultured for 6–8 days at 37° (in a CO_2 incubator) in DMEM containing 10% heat-inactivated foetal calf serum (FCS, Flow) at which time the cultures were confluent and ready to be used to assay the activity of IL-1. Rabbit synovial fibroblasts were isolated from minced normal synovial lining by digestion for 4 hr at 37° with collagenase and protease, in a manner similar to that described for chondrocytes. Isolated cells were grown up in 75 cm^2 flasks (Flow) and confluent cultures were trypsinized and aliquoted into 24 well plates at 10^4 cells per well and grown to confluency at which point they were used to assay the effect of IL-1.

Assay of IL-1 activity on isolated rabbit cells. One day before adding IL-1 to confluent cultures of cells the medium was removed and replaced with DMEM containing 0.5% BSA. This was done to allow culture media to be assayed for neutral metalloproteinase activity, these enzymes being inhibited by serum inhibitors. A range of concentrations of the various preparations of IL-1, diluted in this medium, was then added to the cells which were cultured for a further 24 hr. The media were assayed for the presence of prostaglandin E_2 (PGE_2) as described by Salmon [14]. Briefly, rabbit antiserum to PGE_2 was incubated with test samples and tritiated standard. Free radioactive material was removed with dextran-coated charcoal and the residual bound activity was measured in a scintillation spectrometer.

Intra-articular injection of IL-1. Adult male New Zealand white rabbits (weight 2.5–3.5 kg were used). The various IL-1 preparations were injected through the suprapatellar ligament into the joint space and the contralateral joint received an equal volume of the appropriate vehicle. Recombinant and natural porcine IL-1 molecules were diluted in normal saline containing 0.02% low endotoxin foetal calf serum (Hyclone, UT). The commercial human highly purified IL-1 was used as described by Pettipher *et al.* [4].

Animals were killed 4 hr or 24 hr after a single injection and the joint diameters were measured by calipers. The joint space was then washed with 1 ml of sterile saline and the resultant joint wash fluids from control and IL-1-injected joints were removed for total and differential leukocyte counts and for the assay of the PGE_2 content of the cell-free fluid. Samples of synovial lining were dissected and placed in buffered formalin for subsequent paraffin wax embedding and histological sectioning. The articular cartilage was dissected from the ends of the femurs for the assay of the proteoglycan content.

Assay of the proteoglycan content of articular cartilage. Articular cartilage was dissected from the ends of the femurs from control and IL-1-injected joints, blotted dry, weighed and digested with papain at 65° for 1 hr. The content of sulphated glycosaminoglycans (the heteropolysaccharide side chains of proteoglycans) was measured by the 1,9-dimethylmethylene blue binding assay [15]. The proteoglycan content was expressed as μg of glycosaminoglycan per mg weight of cartilage and the IL-1-injected joint was compared with its contralateral control joint to give the percentage loss of proteoglycan.

Assay of sulphated glycosaminoglycans in joint fluid. Aliquots of joint fluid were digested with papain and reacted with 1,9 dimethylmethylene blue as described by Farndale *et al.* [16].

Endotoxin levels in IL-1 preparations. The concentration of endotoxin in the various IL-1 preparations was assayed by means of the limulus amoebocyte lysate assay [17].

RESULTS

Potency of IL-1 preparations in LAF assay

The commercial preparation of the human natural IL-1 gave results in the LAF assay consistent with the manufacturer's claims. The hr-IL-1α and β were equipotent with an approximate activity of 10 LAF units/ng. The porcine IL-1β showed no LAF activity over the concentration range 0.5–20 ng/ml. In contrast the porcine IL-1α had a LAF activity of 0.5 LAF units/ng.

Activation of rabbit connective tissue cells

All preparations of IL-1 stimulated rabbit synovial fibroblasts and articular chondrocytes to produce PGE_2 in a dose-dependent fashion. This stimulation was inhibited by > 95% by indomethacin (10^{-6} M). With both cell types both forms of pig IL-1 were equiactive as were both human IL-1s. However, it can be seen that the human IL-1 molecules are significantly more active than the porcine confirming the results with the LAF assay. The response of rabbit articular chondrocytes to pig or human IL-1 is shown in Fig. 1.

Inflammatory responses to intra-articular IL-1

(i) *Joint swelling.* A single intra-articular injection of up to 50 ng hr-IL-1 (α or β) or pig IL-1 (α or β) did not produce swelling of the joint when measured 4 hr or 24 hr later. Two injections of hr-IL-1 over a

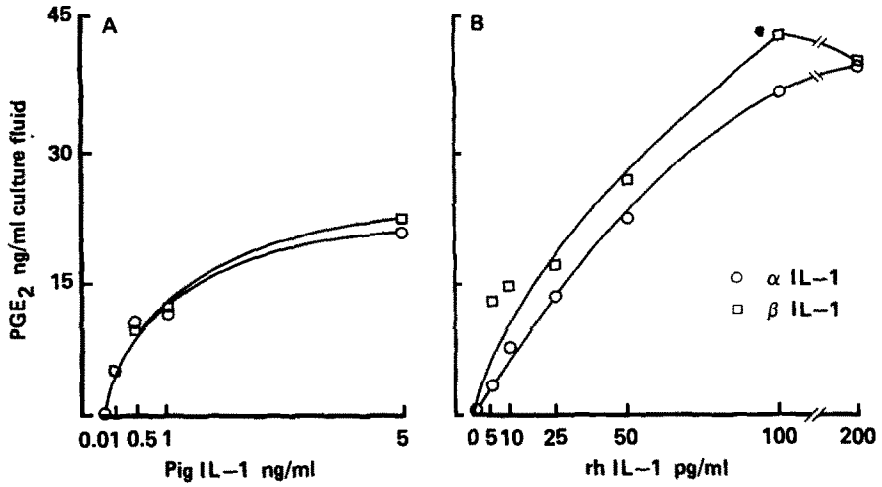

Fig. 1. Stimulation of prostaglandin E_2 (PGE_2) synthesis by monolayer cultures of rabbit articular cartilage chondrocytes exposed to: (A) pig IL-1 (ng/ml) or (B) human recombinant IL-1 (pg/ml). The results are the mean values from triplicate cultures. The error bars have been omitted as they overlap.

6 hr period likewise failed to produce any joint swelling when measured 24 hr after the first injection. Multiple injections of human or pig IL-1 daily for 4 to 6 days respectively also failed to cause swelling of injected joints.

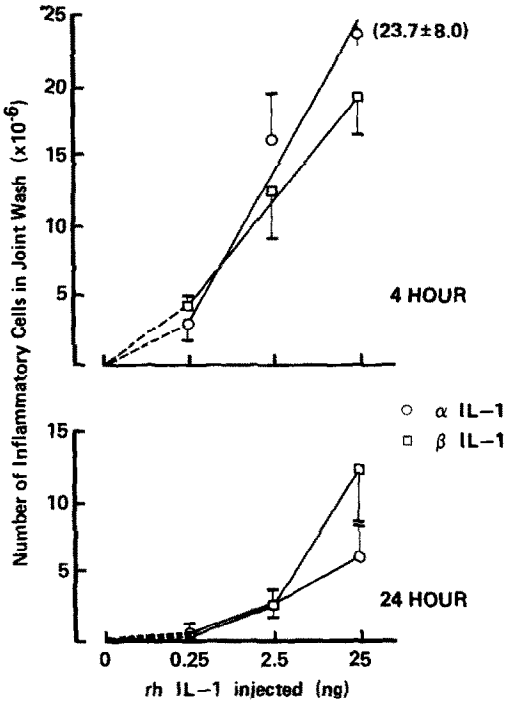

Fig. 2. The number of inflammatory leukocytes in the joint cavity at 4 hr (upper graph) or 24 hr (lower graph) following the injection of 0.25–25 ng hr-IL-1α (○) or hr-IL-1β (□). Each point represents the mean ± SEM of the values from six animals. Injection of vehicle into the contralateral joints induced little cellular infiltration and each point on these graphs is significantly different ($P < 0.01$) from the vehicle control. There is no significant difference between the numbers of cells induced to enter the joint cavity by IL-1α and IL-1β.

(ii) *PGE_2 synthesis*. The concentration of PGE_2 in normal synovial joint wash fluid is below the limit of assay detection (0.05 ng/ml). In rabbits with antigen-induced arthritis the concentration of PGE_2 ranges from 0.5–10 ng/ml [18]. In the joints of rabbits given single or multiple injections of human or pig IL-1 and killed 4 hr or 24 hr after the last injection of cytokine the levels of PGE_2 present were always below the limit of detection.

(iii) *Leukocyte infiltration*. All preparations of natural and recombinant IL-1 were capable of stimulating the dose-dependent accumulation of polymorphonuclear leukocytes (PMN) and monocytes/macrophages in the synovial lining and synovial cavity following a single injection. Injection of the vehicle, a dilution of foetal calf serum in normal saline, reproducibly resulted in the accumulation of $< 10^4$ cells in the joint cavity. The total numbers of infiltrating leukocytes present in the joint cavity 4 hr or 24 hr after injecting 0.25–25 ng hr IL-1 is shown in Fig. 2. There is a dose-dependent increase in cell numbers over this dose range with a mean value of up to 2.4×10^7 cells present in the joint cavity 4 hr after the injection of 25 ng IL-1α. Large numbers of PMN were present in the synovial lining (both in blood vessels and in the stroma of the synovial lining) 4 hr after injection of IL-1. By 24 hr there were fewer PMN visible but infiltrating mononuclear cells were evident. There were no quantitative or qualitative differences in the cell accumulation induced by these recombinant IL-1 molecules. At 4 hr the predominant leukocyte in the joint was the PMN. By 24 hr the proportion of PMN had decreased and the infiltrate contained approximately equal numbers of PMN and monocytes/macrophages with only a few percent of lymphocytes (Table 1). The detailed kinetics of PMN and monocyte/macrophage accumulation in the joint cavity following the injection of 25 ng hr IL-1α is shown in Fig. 3.

Comparing the *in vivo* activities of natural and recombinant human IL-1 by regression analysis it appears that 10 units of natural IL-1 (Genzyme) as reported in [4] induces an equivalent response to 1–

Table 1. Differential count of cellular infiltrate in the synovial cavity 4 hr or 24 hr after intra-articular injection of human natural IL-1 (10U) or human recombinant IL-1s (25 ng)

IL-1	4 hr PMN	4 hr Lymph*	4 hr Mono†	24 hr PMN	24 hr Lymph	24 hr Mono
(5) Natural	86 ± 1	0	14 ± 1	(2) 30	3	67
(3) αIL-1	90 ± 11	0	11 ± 11	48 ± 7	2 ± 3	50 ± 9
(3) βIL-1	96 ± 3	0.3 ± 0.6	4 ± 3	50 ± 24	2 ± 4	48 ± 25

* Lymph — lymphocytes.
† Mono — monocytes/macrophages.
( ) — numbers of animals used.

2 ng recombinant IL-1α and IL-1β. This is consistent with our *in vitro* data which indicates that the hr-IL-1 molecules have activities of around 10 units/ng.

Both pig IL-1α and IL-1β induced approximately equivalent leukocyte accumulation although they were less active than human IL-1 on a weight basis (results not shown).

Cartilage proteoglycan degradation

All preparations of IL-1 were capable of inducing loss of proteoglycan from articular cartilage after a single intra-articular injection. Pig IL-1α and IL-1β, at a dose of 50 ng, produced a significant ($P < 0.05$) reduction in cartilage proteoglycan content of 23.2 ± 3.3 and 16.5 ± 6.9% (mean ± SEM; N = 4) respectively. This reduction in the proteoglycan content was reflected in the dose-related elevation of proteoglycan fragments in the joint fluid (Fig. 4).

The dose-related loss of proteoglycan from cartilage induced by hr IL-1α or hr IL-1β is shown in Fig. 5 with both molecules having a similar effect. The reduction in cartilage proteoglycan content in response to these recombinant molecules was also reflected by the appearance of proteoglycan fragments in the joint fluid. The levels of sulphated proteoglycans were significantly ($P < 0.001$) increased from less than 15 μg/ml in control joint fluids to 68.7 ± 15.9 μg/ml (mean ± SEM; N = 4) and 101.5 ± 12.0 μg/ml (N = 4) 24 hr after intra-articular injection of 25 ng IL-1α or IL-1β respectively.

DISCUSSION

We have previously demonstrated that the intra-articular injection of highly purified natural human IL-1 (Genzyme) in the rabbit causes the accumulation of inflammatory leukocytes in the joint and the loss of proteoglycan from articular cartilage [4]. This preparation of IL-1 from activated human monocytes contains predominantly IL-1β [5, 8]. Since the discovery that two genetically distinct IL-1s are produced by cells it has been determined that these chemically distinct molecules are equipotent in

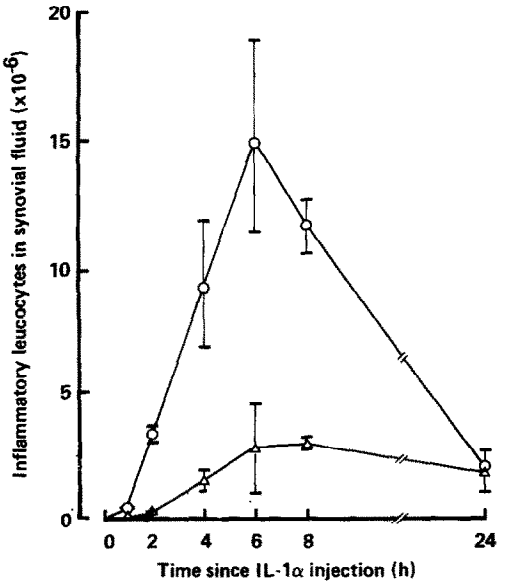

Fig. 3. The total numbers of polymorphonuclear leukocytes (○) or monocytes/macrophages (△) in the joint cavity at various times up to 24 hr after injecting 25 ng IL-1α. Each point is the mean ± SEM of 3–10 animals.

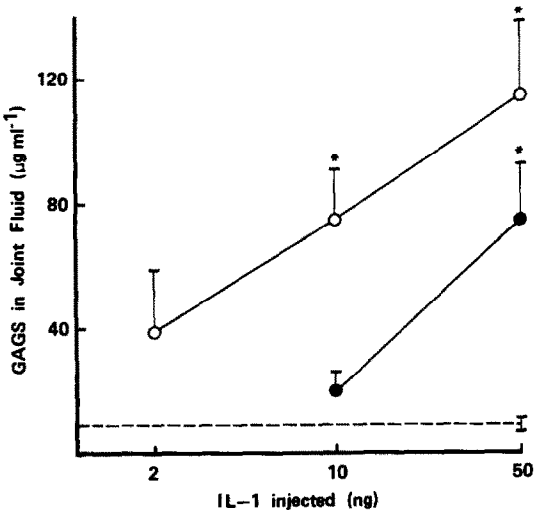

Fig. 4. The concentration of glycosaminoglycans (GAGS) in the synovial joint wash fluid from rabbits 24 hr after the intra-articular injection of porcine IL-1α (○) or IL-1β (●). Each point is the mean ± SEM of 4–10 animals. The dotted line is the mean value ± SEM of the glycosaminoglycan content of the vehicle-injected contralateral joints (* $P < 0.01$ compared to the contralateral control joints).

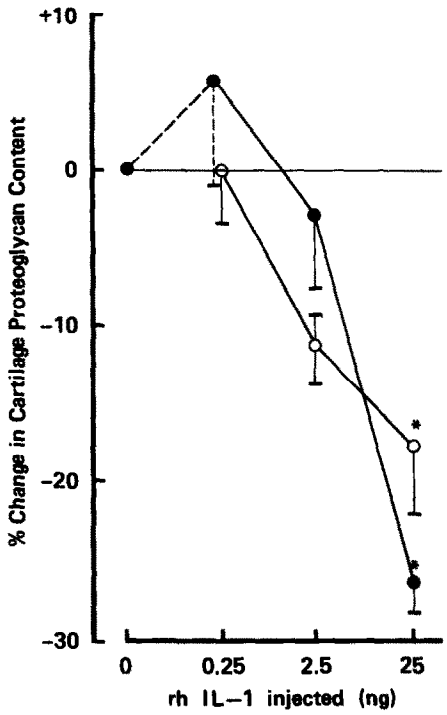

Fig. 5. The loss of proteoglycan from the articular cartilage of rabbit knee joints injected with 0.25–25 ng hr-IL-1α(○) or hr-IL-1β(●). The results are expressed as a percentage loss compared with the contralateral joints which were injected with vehicle. Each point is the mean ± SEM of value from six animals (* $P < 0.01$ compared to contralateral control joints).

a number of *in vitro* assays [19]. However, in other *in vitro* assays these natural or cloned forms of the IL-1 molecule have been reported to demonstrate different potencies [7–9]. Furthermore, IL-1β, but not IL-1α, is reported to stimulate adreno-corticotrophic hormone release in conscious, freely moving, rats [20]. On the basis of these reported differences in the potency of IL-1α and IL-1β, it was of interest to compare the response in the rabbit synovial joint to the intra-articular injection of these two forms of IL-1.

In preliminary studies it was demonstrated that both the neutral and acidic pI forms of human IL-1 were capable of stimulating rabbit articular chondroyctes and synovial fibroblasts to synthesize and secrete PGE_2. Both forms of pig IL-1 were equiactive in these assays as were both forms of human IL-1. However, comparison of the activity of pig and human IL-1 revealed that the latter was more active on a weight basis.

Injection of porcine or human IL-1α or IL-1β into the rabbit knee produced a dose-dependent accumulation of inflammatory leukocytes in the synovial lining and synovial cavity. Again the human IL-1s were more potent than the porcine molecules. Comparison of the α and β forms from both species revealed that they were approximately equipotent in stimulating cellular accumulation. Human IL-1 is remarkably active in stimulating the accumulation of inflammatory leukocytes into various organs and tissues of the rabbit [4, 21, 22]. At 4 hr following the injection of 25 ng of hr-IL-1 into the joints of 3 kg rabbits there was an average of 2.4×10^7 ($N = 6$) leukocytes in the joint cavity. Analysis of the kinetics of leukocyte entry into the joint revealed that measurable numbers of PMN were present in the synovial lining and joint cavity 1 hr after injecting 25 ng IL-1α. At 2 hr the joint cavity contained a mean number of 3×10^6 PMN. The infiltration of PMN peaked at between 4 and 8 hr and rapidly declined thereafter. In contrast the infiltration of the joint by monocytes had a slower time course reaching a plateau at 6 hr.

The levels of leukocyte accumulation induced at 24 hr by injecting 2.5 or 25 ng recombinant IL-1 is comparable to that observed 24 hr after antigen challenge in the Dumonde-Glynn model of antigen-induced arthritis. This model in the rabbit closely resembles rheumatoid arthritis [23, 24]. However, in contrast to this experimental arthritis, the leukocyte accumulation in IL-1-injected joints was not associated with swelling of the joints nor with the appearance of elevated levels of PGE_2 in the joint fluid. There is an obvious relationship between the production of PGE_2 in the joint and joint swelling as exemplified by the inhibition of swelling by non-steroidal anti-inflammatory drugs [25]. This failure to induce PGE_2 production *in vivo* is surprising as both natural and recombinant IL-1 induce the synthesis of prostanoids by a variety of cells in culture including synovial fibroblasts [26], articular chondrocytes [27] and vascular endothelial cells [28]. Moreover, injection of ng quantities of recombinant IL-1α systemically into rabbits produces a monophasic fever [29] and this pyretic response to IL-1 can be blocked by ibuprofen, a non-steroidal anti-inflammatory drug [30]. Thus IL-1 given intravenously is able to induce PGE_2 production in the preoptic hypothalamus. The failure of single or multiple injections of IL-1 given locally to stimulate PGE_2 production in the joint remains unexplained. The implication is that joint tissues have endogenous control mechanisms operating to inhibit prostanoid production induced by IL-1 and that the stimulation of PGE_2 synthesis by joint tissues *in vivo* requires multiple signals.

In addition to the accumulation of inflammatory leukocytes in the joint all preparations of IL-1 were capable of inducing the loss of proteoglycan from articular cartilage. This activity could be assessed either directly or by assaying the synovial fluid for the presence of proteoglycan fragments. Again, as with leukocyte accumulation, both forms of the pig or of human IL-1 were equiactive in promoting proteoglycan loss from cartilage. Comparison of the activity of the pig and human molecules on a weight basis revealed that they were approximately equipotent in causing cartilage breakdown. The loss of proteoglycan 24 hr after injecting 25 ng hr-IL-1 into 3 kg rabbits was equivalent to that in rabbits with antigen-induced arthritis of 1 week's duration [4].

Interleukin 1 activity is produced by human [31] and porcine synovial lining tissue [32] and is found in the joint fluids of patients with chronic arthritis [3]. The composition of the IL-1 produced by joint

tissues has not been established. The present study demonstrates that both IL-1α and IL-1β would contribute equally to disease pathology. These studies further suggest that IL-1 is not the major stimulus to the presence of PGE_2 in the inflamed joint and that other factors must be involved in stimulating the pathways of prostanoid formation.

Acknowledgements—We thank Anita Caffery for excellent technical assistance.

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