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Experimental arthritis induced by continuous infusion of IL-8 into rabbit knee joints

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SUMMARY

To determine the roles of IL-8 in inflammatory synovitis, examination was made of the results of continuously injecting human recombinant IL-8 into the knee joints of New Zealand white rabbits. Recombinant human IL-8 was infused continuously into the joint cavity at 75 ng/h for 14 days by a polypropylene catheter connected to a mini-osmotic pump implanted in each rabbit. Infiltration of inflammatory cells into joint cavity and histopathological changes in synovial tissue were examined at 7 and 14 days following the start of infusion. The continuous infusion of IL-8 for 14 days led to severe arthritis characterized by apparent erythema and joint pain, the accumulation of leucocytes, infiltration of mononuclear cells in synovial tissue, and marked hypervascularization in the synovial lining layer. IL-8 may be a factor which can contribute to the inflammatory process of chronic arthritis by mediating leucocyte recruitment and hypervascularization in inflamed joints.

Keywords cytokines animal models rheumatoid arthritis

INTRODUCTION

The infiltration of neutrophils into inflamed joints in rheumatoid arthritis (RA) has been well studied. Neutrophils are predominant cells in inflammatory synovial fluid [1]. Although neutrophils are less frequently found in inflamed synovial tissue of RA, the apparent infiltration of neutrophils has been demonstrated in the synovial tissue of acute and severe forms of RA. Recruitment of neutrophils through postcapillary venules may result from various chemotactic factors such as C5a formed by complement activation, leukotriene B4 and cytokines produced within inflamed joints. Infiltrated neutrophils secrete various proteolytic enzymes, eicosanoids and the superoxide anion [2]. Recruitment of neutrophils into inflamed joints may thus contribute to tissue damage in RA.

A novel cytokine, IL-8 or neutrophil-activating peptide 1 (NAP-1), has recently been identified [2]. IL-8 is a basic, heparin binding polypeptide consisting of 72 amino acids, and belongs to a new chemotactic cytokine family (chemokine). IL-8 is not only a chemoattractant for neutrophils, T cells [3] and basophils [4], but also an activator of neutrophils. IL-8 stimulates the degranulation and respiratory burst of neutrophils [2].

IL-8 has been identified in synovial fluid and synovial tissue of RA [5]. *In situ* hybridization and immunohistochemical analysis show macrophages and fibroblast-like synoviocytes in rheumatoid synovium to express and secrete IL-8 [6]. Fibroblast-like synoviocytes are capable of producing IL-8 in response to IL-1 and tumour necrosis factor (TNF) *in vitro* [7]. IL-8 synthesis thus occurs in inflamed joints and may contribute to the infiltration of neutrophils into rheumatoid inflamed joints.

The intra-articular injection of human recombinant IL-8 (rIL-8) into rabbit joints was carried out to determine whether IL-8 functions as a mediator of neutrophil recruitment into joints. A single injection of rIL-8 into the knee joints of New Zealand white rabbits resulted in acute arthritis [5]. It caused erythema and joint pain, and facilitated the migration of neutrophils into the joint cavity and synovial tissue, and morphological changes in the synovial lining. IL-8 appeared to have a self-limiting effect on synovial inflammation. The continuous administration of IL-8 into joints for long periods may be necessary for determining the pathological role of IL-8 in chronic arthritis. Human rIL-8 was thus infused into joint cavities using an implanted micro-osmotic pump in the present study.

The continuous infusion of IL-8 for 2 weeks caused apparent erythema and joint pain, leucocyte infiltration and hypervascularization.

MATERIALS AND METHODS

Reagents

Human recombinant IL-8 (2×10^6 U/mg; neutrophil chemotaxis assay [8]) was kindly provided by Dainippon Pharmaceutical

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Co. (Osaka, Japan), and contained less than 0·06 ng endotoxin/μg of protein by limulus amoebocyte lysate assay. Carrier-free human rIL-8 was diluted in Hanks' balanced salt solution containing 1% normal rabbit serum (HBSS-rabbit serum). An Alzet mini-osmotic pump (model 2002; Alza, Palo Alto, CA), with a 200 μl reservoir and delivery rate of 0·5 μl/h, was used. Each sterilized pump was filled with 200 μl of rIL-8 solution at 150 μg/ml or 200 μl of control vehicle (HBSS-rabbit serum). rIL-8 was injected at a rate of 75 ng/h.

Continuous infusion of IL-8

Adult male New Zealand white rabbits (weight 3·0–3·5 kg) were used. Each animal was housed in a standard wire-bottomed cage and allowed water and food *ad libitum*. For implantation of the Alzet mini-osmotic pump, each rabbit was anaesthetized with pentobarbital sodium (Nenbutal; 30 mg/kg body weight) in an amount sufficient to keep the animals in a state of unconsciousness for 3–4 h. A medial incision above the knee joint and puncture of the suprapatellar pouch were made. A polypropylene catheter connected to a pump was inserted through the suprapatellar pouch into the knee joint cavity. The pump was implanted subcutaneously into the medial area of the femur. The tubing and pump were fixed tightly in place with a few stitches. After continuous infusion for 7–14 days, the animals were killed by the administration of Nenbutal. Joint diameter was measured by a caliper just before killing. The joint space was washed twice with 1 ml of HBSS. Injection was conducted with a 26 G needle and syringe through the suprapatellar ligament. After manipulating the joint gently to mix its contents, joint fluid from the control and IL-8-infused joints was carefully collected and centrifuged for 3 min at 3000 ***g***. The infiltrated cells were resuspended in HBSS, and the total cell number of leucocytes, after staining with Türk solution, was determined using a haemocytometer. This was followed with Wright–Giemsa solution staining for differential leucocyte count.

Light microscopic analysis

At 7 and 14 days following the continuous infusion of IL-8 or the control vehicle, synovial tissue was dissected for histopathological examination from the anterior synovium on either side of the patella and infrapatellar synovium. Tissue samples were fixed in neutral buffered formalin and embedded in paraffin wax. Each thin section (3–4 μm) was stained with haematoxylin and eosin and examined for cellular infiltration, synovial membrane thickening and vascularity by light microscopy.

Quantitative histological analysis was performed as described previously [9]. In brief, the numbers of leucocytes and vessels were counted in 50 random microscopic fields using a ×400 objective. The numbers represented mean values ± s.d.

RESULTS

Clinical manifestation of continuous IL-8 injection

Clinically severe erythema and limping became apparent in treated animals within 2 or 3 days after the start of the continuous infusion of IL-8, and continued to be evident throughout the study. No appreciable increase in diameter of a treated joint could be detected. A single injection of 5 μg human rIL-8 into a knee joint has been shown to rapidly cause the same clinical manifestations within a few hours. Continuous infusion of control vehicle caused no clinically apparent inflammatory response during 14 days.

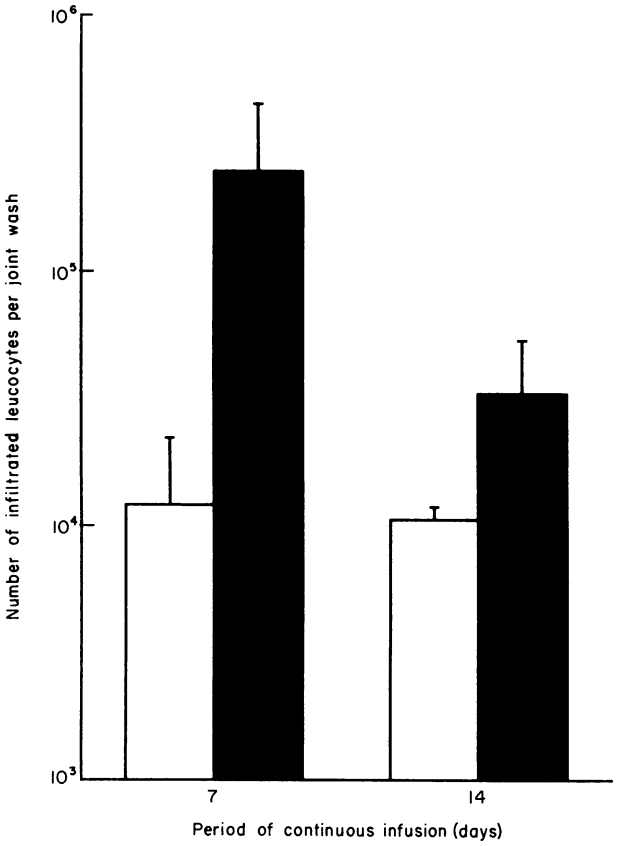

Fig. 1. Leucocyte accumulation in the joint cavity induced by continuously infused human rIL-8 at 75 ng/h for 7 or 14 days. The numbers of cells in joint wash fluid were counted for the IL-8-infused joint (■) and vehicle-infused joint (□) at 7 and 14 days after the start of infusion and expressed as means ± s.d. of triplicate experiments.

Kinetics of leucocyte recruitment into the joint cavity

As shown in Fig. 1, rIL-8 caused marked infiltration of leucocytes into the joint space even at 7 and 14 days after the start of the experiment. The degree of infiltration was more prominent at day 7 than at day 14. The control vehicle caused only slight infiltration, being only 5% of that by IL-8 on day 7.

Differential leucocyte count indicated neutrophils to be predominant (73% at day 7 and 64% at day 14) among accumulated cells. Most mononuclear cells that infiltrated the joint cavity were small, round and negative for α-naphtyl butyrate esterase staining.

A single injection of 5 μg human rIL-8 into a knee joint has been shown to cause leucocyte infiltration into the joint cavity in a time-dependent, self-limiting manner. It induced the rapid accumulation of neutrophils, followed by the migration of mononuclear cells. The continuous infusion of low dose rIL-8

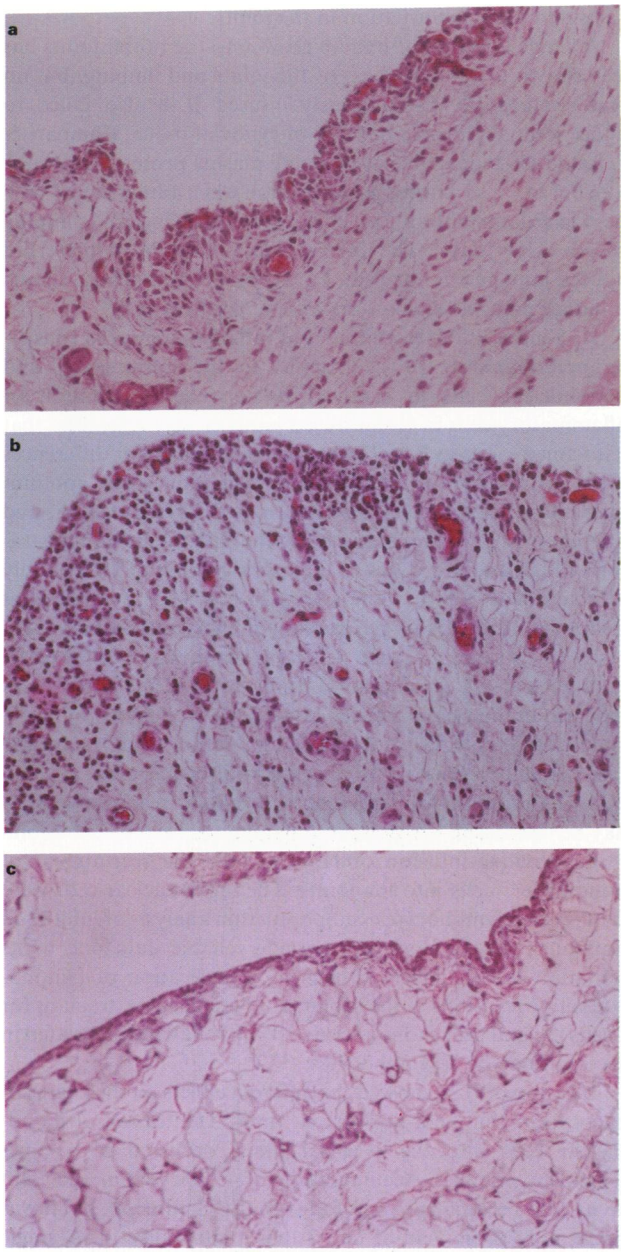

Fig. 2. Cellular infiltrate and synovial neovascularization induced by IL-8 in the joint space. Synovial tissue was obtained from IL-8-infused joints at day 7 (a) or 14 (b) and from vehicle-infused control joints at day 14 (c) (original magnification × 200). (a) Synovial tissue at 7 days after continuous infusion of IL-8, showing the accumulation of a large number of mononuclear cells about the synovial lining layer. Synovial lining cells are multilayered. Vessels in the synovial lining layer are congested. (b) Synovial tissue at 14 days after continuous infusion of IL-8, showing the accumulation of an increased number of mononuclear cells about the superficial layer and around vessels in the synovial lining layer. Vessels in synovial tissue have increased prominently. Many vessels are dilated and congested with erythrocytes. (c) Synovial tissue at 14 days after continuous infusion of the vehicle, showing only mild fibrinous change in the superficial synovial layer. Recruitment of inflammatory cells and angiogenic response are not apparent.

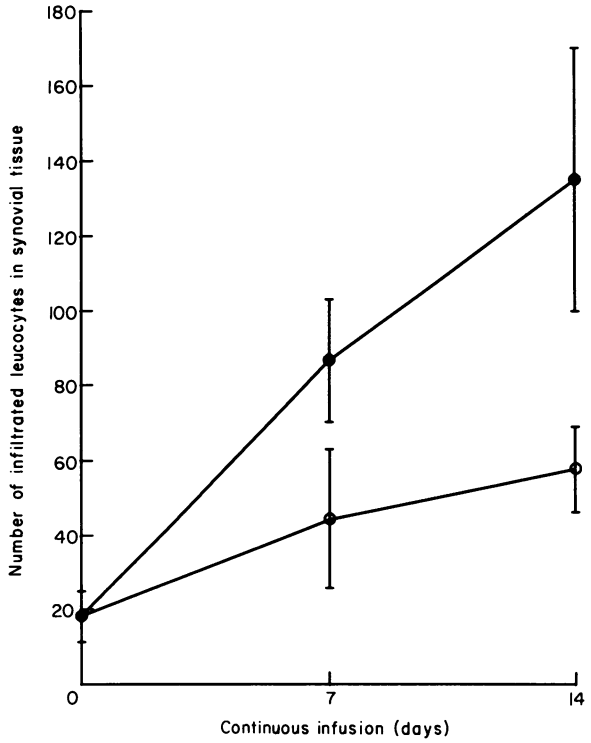

Fig. 3. Kinetics of the accumulation of leucocytes in synovial tissue induced by the intra-articular infusion of IL-8. Human recombinant IL-8 was infused continuously by a mini-osmotic pump into the joint cavity and infiltration of leucocytes in synovial lining layer was determined quantitatively for IL-8 (●) and vehicle-infused joints (○). Each point represents the mean ± s.d. of triplicate experiments. Continuous infusion of IL-8 significantly increased leucocyte accumulation at day 14 ($P < 0.05$).

(75 ng/h) brought about neutrophil infiltration predominantly throughout the experimental period.

Production of antibody to infused human IL-8 may modulate the effect of IL-8. Thus, specific antibody to IL-8 was determined in serum and synovial fluid of rabbits obtained at day 14 by double-immunodiffusion assay. Neither antibody to human IL-8 nor antibody to rabbit IL-8 was detected.

Histological effects due to continuous IL-8 injection

The synovial tissue of normal joints is a thin layer of synovial lining cells underlying loose connective tissue. Only a small number of vessels are present in the normal synovial lining layer.

Tissue sections prepared on days 7 and 14 were examined. The infiltration of inflammatory cells about the synovial lining layer of synovium could be seen on day 7 (Fig. 2a). The marked infiltration of leucocytes was also observed in the synovial lining layer, particularly around many vessels in the synovium dissected on day 14 (Fig. 2b). The recruited inflammatory cells consisted primarily of mononuclear cells and only a small number of neutrophils. Mononuclear cells in the synovium were small and round without large vacuoles, and negative for α-naphtyl butyrate esterase staining, thus showing them to possibly be lymphocytes.

The number of vessels in the synovial lining and subsynovial connective tissue increased on days 7 and 14 (Fig. 2a, b). These vessels were dilated with marked congestion.

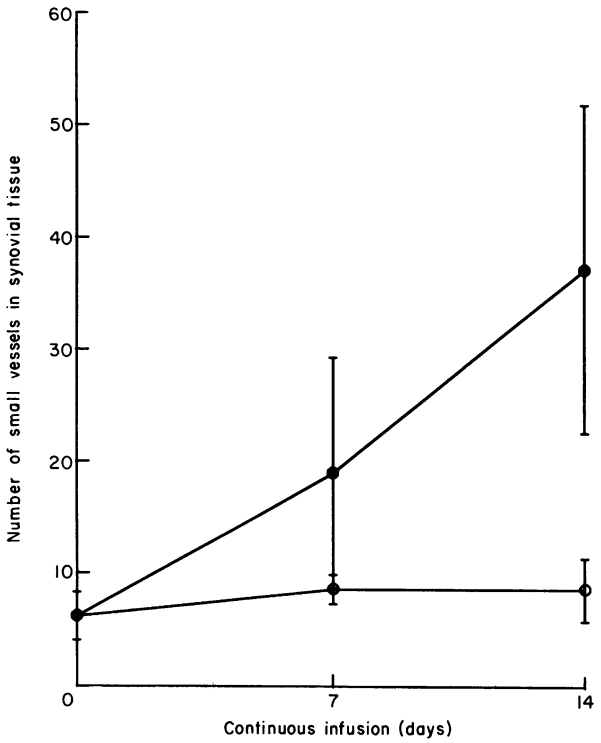

Fig. 4. Kinetics of neovascularization in synovial tissue induced by IL-8 infusion. The number of vessels in synovial tissues was determined quantitatively for IL-8-infused (●) and vehicle-infused joints (○) for the periods indicated. Each point represents the mean ± s.d. of triplicated experiments. The intra-articular continuous infusion of IL-8 significantly increased the number of vessels in synovial tissue at day 14 ($P < 0.05$).

Neither the accumulation of synovial fibroblasts nor formation of a pannus could be detected in IL-8-infused joints. There was no evidence of morphological change in articular cartilage.

In the vehicle-infused joints, leucocyte accumulation and hypervascularization were not found even at day 14. Fibrinous components appeared to have increased in the superficial layer of the synovial lining (Fig. 2c).

For quantitative examination of histological changes, infiltrated leucocytes and vessels were counted in 50 random microscopic fields (×400) in bands of tissue 250 μm wide from synovial lining cells. As shown in Fig. 3, the continuous infusion of IL-8 increased the number of infiltrated leucocytes consisting predominantly of mononuclear cells in a time-dependent manner. Significant differences in the numbers of infiltrated cells of synovial tissue of joint infused by IL-8 and control vehicle for 14 days ($P < 0.05$) were noted. IL-8 infused for 14 days caused a significant increase in the number of vessels in the synovial tissue ($P < 0.05$) (Fig. 4). IL-8 is thus shown to induce the recruitment of inflammatory cells and hypervascularization in synovial tissue.

DISCUSSION

The capacity of IL-8 for initiating synovial inflammation was assessed following the continuous intra-articular infusion of human recombinant IL-8 into the joint space of rabbit knee joints. Infusion for 14 days caused erythema and joint pain, recruitment of inflammatory cells and marked hypervascularization. There was no joint swelling or accumulation of fibroblasts and pannus formation in the joints.

A single injection of human rIL-8 into the rabbit joints has been shown to cause redness of the joints and limping, but no joint swelling [5]. Continuously infused IL-8 also failed to induce joint swelling or oedema of synovial tissue. Rampart *et al.* found IL-8 incapable of inducing plasma protein extravasation, but to cause marked extravasation when administered with the vasodilator substance, PGE_2 [10]. IL-8 induced limping within a few days. The precise mechanism by which IL-8 induced joint pain is not clear, but IL-8 has recently been shown to induce pain through a sympathetic pathway [11].

IL-8 induced the recruitment of inflammatory cells consisting predominantly of neutrophils in the joint cavity and mononuclear cells in synovial tissue. The distribution of inflammatory cells in IL-8-infused joints closely resembles that in inflamed joints in RA. The precise mechanism for differences in the distribution of inflammatory cells induced by continuously infused IL-8 has yet to be determined. Larsen *et al.* showed differences in cell types attracted to the skin by the administration of high and low doses of IL-8. A high dose of IL-8 mainly attracted neutrophils, while a low dose of IL-8, lymphocytes predominantly [3]. A single injection of human recombinant IL-8 into joints rapidly caused neutrophil infiltration followed by mononuclear cell accumulation. Infiltration of neutrophils was apparent in the synovial tissue at 4 h following injection, but it could not be detected at 24 h. Accumulation of mononuclear cells could be observed in the synovial tissue even at 24 h following injection [5]. Differences in cellular response to IL-8 may be the reason for those in inflammatory cell distribution.

Continuous infusion of IL-8 induced the recruitment of mononuclear cells into the joints. The mononuclear cells were small, round lymphocytes. Subpopulation analysis of infiltrated mononuclear cells does not provide reliable data due to its limitation in immunohistochemical identification of lymphocyte subpopulations in the rabbit. IL-8 is a chemoattractant for T cells [3], and thus mononuclear cells that have accumulated in the joint may be T lymphocytes.

Infusion of IL-8 showed the different effects on the degree of leucocyte recruitment at different times. Infiltration of mononuclear cells into the synovial tissue occurred in a time-dependent manner. Accumulation of leucocytes in the joint cavity also became apparent following IL-8 infusion, but it was much more prominent at day 7 than that at day 14. A single injection of IL-8 caused only a transient accumulation of leucocytes in the joint [5]. These data indicate continuous infusion of IL-8 may attract leucocytes continuously.

Neovascularization is a prominent feature in the development of RA. Various factors such as fibroblast growth factor (FGF) [12] and transforming growth factor-beta (TGF-β) cause angiogenic responses *in vitro* and *in vivo* [13]. Macrophages and fibroblast-like synoviocytes in rheumatoid synovial tissue are major sources of angiogenic mediators [14]. Koch *et al.* showed IL-8 to be capable of inducing an angiogenic response *in vivo* and enhancing migration and the proliferation of endothelial cells *in vitro*. The chemotactic activity of endothelial cells in conditioned media of rheumatoid synovial macrophages was partially abolished by treatment of the antibody for human IL-8 [15]. In this study, the continuous infusion of IL-8 for 14 days significantly increased the number of vessels in synovial tissue.

These vessels were apparently dilated with congestion. A single injection of IL-8 could not cause histological changes of vessels in the synovial tissue. It is not clear whether the continuous infusion of IL-8 caused angiogenesis or dilatation of pre-existing small vessels in rabbit synovial tissue. However, these observations indicate that continuous infusion of IL-8 for long periods not only recruits inflammatory cells but also induces hypervascularization in synovial tissue.

IL-1 is essential to the development of inflammatory arthritis. Feige *et al.* reported that continuous infusion of human recombinant IL-1α at 200 ng/day for 14 days caused severe arthritis [16]. Serous exudation and neutrophil infiltration as a feature of acute arthritis, as well as pannus formation and cartilage and bone erosion as a feature of chronic arthritis, were observed. IL-1 does not express chemotactic activity toward leucocytes. Neutrophil accumulation into rabbit joints induced by a single intra-articular injection of human IL-1α is completely abolished by a co-injection of antibody for rabbit IL-8 [17]. It thus follows that the recruitment of neutrophils in IL-1-induced acute arthritis is mediated by the endogenous production of IL-8. In this study, the continuous infusion of IL-8 caused prominent leucocyte infiltration. IL-8 thus may contribute to the development of inflammatory responses even in chronic arthritis.

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