The Role of an Epithelial Neutrophil-Activating Peptide-78-Like Protein in Rat Adjuvant-Induced Arthritis

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Peptide-78-Like Protein in Rat Adjuvant-Induced Arthritis 1

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The chemokine, epithelial neutrophil-activating peptide-78 (ENA-78), is a potent neutrophil chemotaxon whose expression is increased in inflamed synovial tissue and fluid in human rheumatoid arthritis compared with osteoarthritis. Since ENA-78 has been implicated in the pathogenesis of RA, we examined the expression of an ENA-78-like protein during the development of rat adjuvant-induced arthritis (AIA). Using an ELISA assay, we found increased levels of antigenic ENA-78-like protein in the sera of AIA animals compared with control normal animals by day 7 postadjuvant injection. ENA-78-like protein levels continued to increase as AIA developed. ENA-78-like protein levels in joint homogenates were increased in AIA animals later in the development of the disease, by day 18 during maximal arthritis, compared with control animals. Expression of ENA-78-like protein in both the AIA serum and joint correlated with the progression of inflammation of the joints. Anti-human ENA-78 administered before disease onset modified the severity of AIA, while administration of anti-ENA-78 after clinical onset of AIA did not modify the disease. These data support a role for an ENA-78-like protein as an important chemokine in the progression and maintenance of AIA. The Journal of Immunology, 1999, 162: 7492–7500.

Rheumatoid arthritis (RA) 3 is an inflammatory disorder characterized by infiltration of leukocytes into the synovial tissue (ST) and synovial fluid (SF) of joints (1, 2). The progression of the disease ultimately leads to destruction of cartilage and bone (1, 2). Leukocyte extravasation through the endothelial barrier and their ingress into the ST are important in the pathogenesis of RA (3). The adhesion of leukocytes to the endothelium and subsequent migration through the endothelium is a complex process involving many different molecules acting sequentially (3–5). Several chemotactic cytokines, termed chemokines, have been implicated in RA as neutrophil (PMN) and mononuclear cell recruiters and activators (6). The chemokine superfamily is characterized by four cysteines with two disulfide bridges and has been classically subdivided into subfamilies (6–8). In one subfamily, the first two cysteines are separated by an another amino acid (C-X-C). Another family is characterized by adjacent cysteines (C-C). Recently, the chemokine superfamily has expanded with additional classes of chemokines, the C and the C-X3-C families (9). The functions of chemokines are not limited to leukocyte recruitment and activation, as they have also been implicated in angiogenesis and in having a protective role in HIV infections (10–12).

ENA-78, a C-X-C chemokine cloned in humans, is a PMN chemotactic factor. It is a 8.3-kDa protein with 78 amino acids containing four cysteines positioned identically to those of IL-8 and its homologues (13). ENA-78 activates PMNs by inducing PMN chemotaxis and promoting cytotoxic-free calcium changes and is an angiogenic factor (10, 11, 13, 14). In human disease, similar to other PMN chemotactic factors such as IL-8, C5a, and leukotriene B4, we found ENA-78 to account for a significant portion of RA SF chemotactic activity for PMNs (14). ENA-78 concentrations were significantly greater in RA SFs than SFs from patients with osteoarthritis or other inflammatory and noninflammatory forms of arthritis (14).

Many aspects of inflammation and the molecules involved have not as yet been examined in RA because of the limitations of obtaining multiple sequential tissue biopsies in humans. There are a number of well-established rodent models for human RA, including the rat adjuvant-induced arthritis (AIA) model (15). AIA is similar clinically and pathologically to RA. In AIA, 90–100% of rats develop arthritis within 14 days after adjuvant injection, showing joint histologic changes including leukocyte invasion preceding joint swelling (16). The role of several cytokines, such as IL-1 (17, 18), IL-6 (17, 19), TNF-α (20), and IL-8 (21), has been implicated in the pathogenesis of various animal arthritis models.

In this study, we examined the involvement of an ENA-78-like protein in AIA. We demonstrated that anti-ENA-78 Ab inhibited rat peritoneal PMN recruitment to recombinant human (rh)ENA-78 as well as to LPS in vivo. We determined the expression of this ENA-78-like protein in the serum and joint homogenates of AIA compared with control rats using ELISAs. We then modulated the progression of AIA by treatment with neutralizing Abs against human ENA-78 either before the onset of clinical
symptoms or after establishment of the disease. Immunization after the onset of disease resulted in no significant changes compared with sham-immunized AIA rats. Immunization of the animals with anti-ENA-78 before development of the disease decreased the joint inflammation compared with sham-immunized AIA rats, suggesting that the ENA-78-like protein is an important pathogenic factor in the development of AIA.

Materials and Methods

Intraperitoneal PMN recruitment studies

Fourteen Lewis rats were given an i.p. injection of 10 ng rhENA-78 with 1 ml anti-ENA-78 (rabbit serum) or 1 ml control rabbit serum. Rabbit serum and rhENA-78 were mixed together immediately before injection. Animals were sacrificed after 4 h, and the i.p. cavity of each rat was lavaged with 3 ml PBS + 5 mM EDTA. White blood cell counts, percentage of PMNs, and number of PMNs/ml were determined from differential and hemocytometer counts performed on peritoneal lavages.

In addition, 14 rats received an i.p. injection of 1 mg LPS with 1 ml anti-ENA-78 or 1 ml control rabbit serum. LPNS and rabbit serum were combined immediately before injection. After 6 h, animals were sacrificed and their peritoneal cavities were lavaged, as described above.

Induction of rat AIA

Lewis female rats (100 g) were injected s.c. into the base of the tail with lyophilized Mycobacterium butyricum (0.3 ml; Difco, Detroit, MI) suspended in mineral oil at 5 mg/ml (22). This animal model has been used previously to compare mineral oil control animals with AIA animals (22). In time-course studies, control animals were injected with mineral oil only (0.3 ml).

Arthritis evaluation

The degree of arthritis, indicated by joint swelling, was quantitated by measuring two perpendicular diameters of the joint using a caliper (Lange Caliper; Cambridge Scientific Industries, Cambridge, MA). Joint circumference was calculated using the geometric formula: circumference = \( \pi \times (a^2 + b^2)^{1/2} \), where \( a \) is the antero-posterior diameter and \( b \) is the antero-posterior diameter, as we have done previously (22, 23). In the anti-ENA treatment experiments, severity of arthritis was quantified by scoring each paw on a scale of 0–4 based on joint swelling, erythema, and joint rigidity (24). The articular index was defined as the sum of the scores of all four paws from each animal, with a maximal possible score of 16. AIA affects mostly the hind limbs. For instance, scores on day 24 postadjuvant injection were 2–3 for hind limbs, while front limb scores were typically 1. Animals were scored by observers blinded to the experimental group identity.

Clinical and laboratory follow-up

Body weight evaluation was conducted on a regular basis postadjuvant injection during the time-course studies. At the time of sacrifice, blood was saved for laboratory tests. Erythrocyte sedimentation rate (ESR) was determined by the Westergren method using a Sedilast autozero ESR system (Polymedco, Cortlandt, NY). A total leukocyte count was obtained using a hemacytometer after RBC lysis in 3% acetic acid. Blood smears were prepared, and the percentage of PMNs was evaluated after Diff-Quik (Baxter, Miami, FL) staining. Ankles were obtained, since they were generally the most affected joints. Joints were either embedded in OCT compound (Miles, Elkhart, IN) for immunohistochemical studies or homogenized.

Preparation of joint homogenates

Isolated ankles were placed into a 50-ml conical centrifuge tube containing 3 ml of lysis buffer (2 mM PMSE, 1 mM/ml antipain, 1 \( \mu \)g/ml aprotinin, 1 \( \mu \)g/ml leupeptin, and 1 \( \mu \)g/ml pepstatin A (all from Sigma, St. Louis, MO) in PBS) and homogenized on ice using a motorized homogenizer, followed by sonication for 30 s. The homogenates were centrifuged at 2000 \( \times g \) for 10 min. The supernatants were filtered using millipore filters (0.2 \( \mu \)m) and stored at minus 80°C until analyzed.

Treatment of rats with anti-ENA-78

Neutralizing polyclonal anti-human ENA-78 was produced in our laboratory. Fourteen Lewis rats were given an i.p. injection of 10 mg rhENA-78 with 1 ml anti-ENA-78 (rabbit serum) or 1 ml control rabbit serum. Rabbit serum and rhENA-78 were mixed together immediately before injection. Animals were injected, as described, with adjuvant on day 0. On days 8, 10, and 12 after adjuvant injection, animals were injected i.p. with either 1 ml of 1 mg/ml neutralizing F(ab')2 anti-ENA-78 or 1 ml of 1 mg/ml F(ab')2 control IgG. Animals were sacrificed on day 14 postadjuvant injection, and serum and joint were collected for investigation. In the second group, animals were injected with adjuvant on day 0. After onset of AIA, animals were injected with either 1 ml of 1 mg/ml neutralizing F(ab')2 anti-ENA-78 or 1 ml of 1 mg/ml F(ab')2 control IgG on days 18, 20, and 22 postadjuvant injection. Animals were sacrificed on day 24 postadjuvant injection, and serum and joints were collected for investigation.

Cytokine ELISA assay and data analysis

The ENA-78 concentration in the serum and homogenate samples was determined using a sandwich ELISA (25). Ninety-six-well plates (Nunc, Roskilde, Denmark) were coated with 50 \( \mu l \)/well rabbit anti-human ENA-78 (3.2 \( \mu g/ml \) in 0.6 M NaCl, 0.26 M HCO\(_3\), and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed in PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Nonspecific binding sites were blocked with 2% BSA in PBS (200 \( \mu l \)), and the plates were incubated for 90 min at 37°C. Plates were rinsed (three times) with wash buffer, and 50 \( \mu l \) of undiluted or 10-fold diluted serum or homogenate samples, as well as standard samples were added to the wells in duplicate, followed by incubation for 1 h at 37°C. Plates were washed (four times), and 50 \( \mu l \)/well biotinylated rabbit anti-ENA-78 (6 \( \mu g/ml \) in PBS, pH 7.5, 0.05 Tween-20, 2% FCS) was added for 45 min at 37°C. Plates were washed (four times), 100 \( \mu l \) streptavidin-peroxidase conjugate (100 \( \mu g/ml \); Dako, Carpinteria, CA) was added, and the plates were incubated for 30 min at 37°C. The plates were washed (three times), and 100 \( \mu l \) diaminobenzidine (Sigma, St. Louis, MO) was added. The plates were incubated at 25°C for 6–10 min, and the reaction was terminated with 50 \( \mu l \) of 3% M sulfuric acid solution in wash buffer plus 2% FCS. Plates were read at 490 nm. The ELISA consistently detected ENA-78 concentrations above 50 pg/ml, when compared against a human ENA-78 standard. The ELISA did not detect human IL-1α, IL-1β, IL-1 receptor antagonist, IL-4, IL-6, IL-7, IL-8, granulocyte-chemotactic protein-2, monocyte-chemotactic protein-1, TGF-β, TNF-α, growth-related gene product α, neutrophil-activating peptide 2, lyP-10, or rat cytokine-induced neutrophil chemotractant-1/Gro. ELISAs detecting the quantity of rat IL-1β and rat TNF-α in joint homogenates were purchased commercially (R&D Systems, Minneapolis, MN, and Biosource International, Camarillo, CA, respectively). Each was used in accordance with the recommendation of the manufacturer.

Immunohistochemistry

Rats were sacrificed on day 14 or day 24, and their joints were embedded in OCT (Miles, Elkhart, IN). Sections (8-μm) were cut using a D-profile knife for bone cutting (Leica, Nussloch, Germany). Immunostaining was performed using a Vector ABC Kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Kirkegaard & Perry, Gaithersburg, MD) as a chromogen (22). Sections were fixed in cold acetone for 20 min. Peroxidase peroxidase activity was quenched by incubating slides for 30 min in 0.3% hydrogen peroxide in methanol. All subsequent incubations were performed for 15 min at 37°C in a moist chamber. The tissue sections were pretreated with 50 μl diluted goat serum; incubated with either rabbit anti-rat IL-1β (Cytokine Science, Boston, MA), rabbit anti-mouse TNF-α (Genzyme, Cambridge, MA), or nonimmune serum (negative control); and washed (twice) in PBS. Slides were not incubated with anti-ENA-78 since this Ab did not react immunohistologically with ST in our hands. The slides were incubated with a 1:400 dilution of anti-rabbit biotinylated Ab in PBS/BSA, washed (twice) with PBS, incubated with avidin/biotinylated HRP complex, and washed (twice) with PBS. Slides were then stained with diaminobenzidine tetrahydrochloride substrate for 5 min at room temperature, rinsed in tap water for 2 min, counterstained with Harris’ hematoxylin, and dipped in saturated lithium carbonate solution for bluing. Immunohistochemical staining was accomplished in batches that were run with positive and negative controls as well as experimental comparison groups simultaneously.

Microscopic analysis

Various cell types in the ST, including lining cells, sublining macrophages, PMNs, lymphocytes, and blood vessels, were identified by immunohistochemical staining reactions and/or morphologic features. The number of PMNs, lymphocytes, macrophages, and blood vessels was recorded as the mean ± SE obtained in any of three high power fields (×400) examined per slide.

Immunostaining was graded by a frequency of staining scale (0–100%), in which 0% indicated no staining and 100% showed that all of the cells were immunoreactive, for each of the ST components. Each slide was scanned using a 40×/0.75 objective.
evaluated by a single observer without knowledge of the animal’s experimental group or sacrifice date. Selected sections were analyzed by two additional observers. The percentage of reactive cells for each Ab was analyzed for different time points within each group and compared between experimental and control groups.

**Statistical analysis**

Data were analyzed using a Student’s t test. The log values of data were used for analysis on Fig. 1 due to high interanimal variation. Correlations were performed using a Pearson correlation coefficient. When significant, p values are indicated by an asterisk (p < 0.05) (26).

**Results**

*Rat PMN recruitment into the peritoneum is inhibited by anti-ENA-78*

To determine whether anti-ENA-78 Ab affected inflammation in rats, 10 ng rhENA-78 was used to induce PMN influx into the peritoneal cavity. Upon sacrifice, the peritoneal cavity was lavaged and the number of cells in the lavage was compared between groups. Anti-ENA-78 significantly inhibited the number of PMNs/ml recruited to the peritoneum in lavages when compared with controls (Fig. 1).

Next, we induced rat peritoneal PMN recruitment using LPS, a known stimulus for the production of human ENA-78 protein. LPS (1 mg) was administered via an i.p. injection to rats, and their peritoneal cavities were lavaged at different time points ranging from 4–18 h (data not shown). The greatest infiltration of white blood cells into the rat peritoneum occurred 12 h after LPS injection. Therefore, we next determined whether a portion of this cellular influx could be due to ENA-78-like protein and if rat PMN recruitment induced by LPS could be inhibited by anti-ENA-78. LPS was introduced concomitant with anti-ENA-78 or control rabbit serum, and after 6 h, peritoneal lavages were collected to determine cell counts (Fig. 2). Anti-ENA-78 significantly inhibited LPS-induced rat PMN recruitment, suggesting the neutralization of an endogenous rat ENA-78-like protein that acts to recruit PMNs in vivo.

**Expression of ENA-78-like protein in serum and joint homogenates**

We performed a time-course study of ENA-78-like protein expression in the serum and joints of control normal and AIA animals. Most of the animals (90%) injected with adjuvant developed AIA by day 14 after injection, as determined by joint circumference, ESR, and total peripheral leukocyte count (data not shown). ENA-78-like protein levels in serum were significantly higher in arthritic rats compared with controls starting at day 7 (p < 0.05) and remained significantly increased through day 41 (Fig. 3A). The highest level of ENA-78-like protein in AIA animals by comparison with control was found on day 18 (Fig. 3A).

**FIGURE 1.** Anti-ENA-78 neutralizes a rat chemotactic activity to rhENA-78. Rats were given 10 ng rhENA-78 + 1 ml anti-ENA-78 Ab i.p. or the same dose of rhENA-78 + 1 ml normal rabbit serum (n = 7). Values represent the mean number of PMNs/ml (±SE) in the peritoneal lavage. Stars indicate statistically significant different values between anti-ENA and control animals (p < 0.05).

**FIGURE 2.** Anti-ENA-78 neutralizes an endogenous rat chemotactic activity. Rats were given 1 mg LPS + 1 ml anti-ENA-78 Ab i.p. or the equivalent dose of LPS + 1 ml normal rabbit serum (n = 7). Values represent the mean percentage of PMNs/ml (±SE) counted in the i.p. lavage. Stars indicate statistically significant different values between anti-ENA and control animals (p < 0.05).

**FIGURE 3.** ENA-78-like protein expression in the serum and joint homogenates of animals. Six AIA and six control animals were used at each time point for serum determinations. For joint homogenates, samples were obtained from both ankles of three rats (n = 6 ankles). Values indicate the mean increase of ENA-78-like protein in AIA animals compared with control animals. Serum ENA-78-like protein was increased significantly in AIA compared with control animals between days 7 and 41 postadjuvant injection (p < 0.05). AIA joints contained significantly more ENA-78-like protein than did control joints after day 18 (p < 0.05). Values indicate the mean increase of ENA-78-like protein in AIA animals compared with control animals. Stars indicate statistically significant different values between control animals and AIA animals (p < 0.05).
ENA-78-like protein expression in the joint extracts was also significantly greater in AIA animals compared with control animals, but occurred later in the course of AIA (Fig. 3B). ENA-78-like protein levels were significantly increased on day 18 postadjuvant injection ($p < 0.05$) (Fig. 3B). The peak level of ENA-78-like protein over controls was reached on day 41 in AIA joints (Fig. 3B). On day 47 of the study, ENA-78-like protein levels remained high in the joints of AIA animals and were significantly higher than in the joints of control animals ($p < 0.05$) (Fig. 3B).

ENA-78-like protein serum levels were positively correlated with the ENA-78-like protein levels in the joints ($r = 0.87; p < 0.05, n = 7$). Significant positive correlations were determined between joint circumference and both joint ENA-78-like protein ($r = 0.78; p < 0.05, n = 7$) and serum ENA-78-like protein concentrations ($r = 0.96; p < 0.05, n = 7$).

**Assessment of anti-ENA-78 treatment before onset of AIA**

Animals were injected with anti-ENA-78 or control IgG on days 8, 10, and 12 postadjuvant injection. Based on the evaluation of ENA-78-like protein expression during the development of the disease, anti-ENA-78 was administered to adjuvant-injected animals before ENA-78-like protein was significantly increased in the joint, but was already being significantly expressed in the serum. When the animals were sacrificed on day 14 postadjuvant injection, the number of peripheral blood leukocytes, the percentage of peripheral blood PMNs, and ESR were determined in the serum. There were no significant differences detected in peripheral blood leukocyte counts, percentage of PMNs, ESR, or body weight between control IgG- and anti-ENA-78-treated animals (data not shown).

There was a decrease in severity of arthritis, as determined by measurement of the joints in anti-ENA-78-treated animals. The four joints (front and hind limbs) were measured as described and their sum totaled. The anti-ENA-78-treated animals had a significantly lower sum circumference than did control IgG-treated animals ($168 \text{ mm} \pm 10$ for control IgG vs $157 \text{ mm} \pm 5$ for anti-ENA-78; $p < 0.05$) (Fig. 4A). The articular index for anti-ENA-78...
animals trended toward a decrease compared with IgG-injected controls, but did not attain statistical significance (3.9 ± 2.3 for control IgG vs 2.2 ± 1.5 for anti-ENA-78) (Fig. 4B). We found a significant positive correlation between joint circumference and articular index ($r = 0.86; p < 0.05, n = 10$).

Histopathology of hind limb ankle joints was performed to determine the composition of the cellular infiltrate in the joints. The hind limb ankle joints were used because they were the most affected. The number of macrophages, lymphocytes, PMNs, and blood vessels for the hind limb ankle joints was determined (Table I). The joints from anti-ENA-78-treated animals displayed decreased mean numbers of PMNs compared with control IgG-treated animals (13 ± 6 vs 2 ± 10 cells/3 high power fields), although these values did not attain statistical significance. There was no significant difference detected for these cell counts between anti-ENA-78-treated and control IgG-treated animals.

Since it is possible that treatment with anti-ENA-78 would reduce expression of other joint cytokines, we performed ST immunohistochemistry for IL-1$\beta$ and TNF-$\alpha$ (Figs. 5 and 6). Synovial lining cells, which are composed of macrophages and fibroblasts, contained many IL-1$\beta$-immunopositive cells (40 ± 9.1% for control IgG), and this reactivity was reduced with anti-ENA-78 treatment (20.4 ± 12.5%) (Fig. 5A). Expression of IL-1$\beta$ on sublining synovial macrophages was minimal for both control IgG (2.8 ± 0.7%) and anti-ENA-78 treatment (1.5 ± 0.2%) (Fig. 5A). ELISAs performed on ankle homogenates for rat IL-1$\beta$ demonstrated a mean 3-fold reduction with anti-ENA-78 treatment, from 68.5 ± 37.5 pg/ml in IgG-treated control animals to 18.8 ± 8.7 pg/ml in animals treated with anti-ENA-78 Ab (Table II). Synovial lining cells expressed TNF-$\alpha$ (21.3 ± 16.4% for controls), and this reactivity was decreased with anti-ENA-78 treatment (0.5 ± 0.2%) (Fig. 5B). Expression of TNF-$\alpha$ on sublining synovial macrophages, like IL-1$\beta$, was found in the minority of ST macrophages for control IgG (1.8 ± 0.7%) and anti-ENA-78 treatment (1.5 ± 0.7%) (Fig. 5B). TNF-$\alpha$ levels in joint ankle homogenates, as detected by ELISA, were not decreased by anti-ENA-78 treatment when compared with IgG-treated animals ($n = 7$; Table II).

**Table II.** IL-1$\beta$ and TNF-$\alpha$ in ankle homogenates of anti-ENA-78 and IgG-treated control rats as determined by ELISA

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<thead>
<tr>
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<th>Anti-ENA Administered Prior to AIA Onset</th>
<th>Anti-ENA Administered After AIA Onset</th>
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<tr>
<td></td>
<td>IL-1$\beta$ (pg/ml)</td>
<td>TNF-$\alpha$ (pg/ml)</td>
</tr>
<tr>
<td>IgG control</td>
<td>68.5 ± 37.5</td>
<td>5.7 ± 4.0</td>
</tr>
<tr>
<td>Anti-ENA-78</td>
<td>18.8 ± 8.7</td>
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<tr>
<td>$p$ value</td>
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*Values are the mean ± SEM.*
Assessment of anti-ENA-78 treatment after the onset of AIA

Animals were injected with anti-ENA-78 or control IgG on days 18, 20, and 22 postadjuvant injection. When the animals were sacrificed on day 24 postadjuvant injection, number of peripheral blood leukocytes, percentage of PMNs, and ESR were determined in the serum. There were no significant differences detected in the peripheral blood leukocyte counts, percentage of PMNs, ESR, or body weight between control IgG- and anti-ENA-78-treated animals (data not shown).

Control and anti-ENA-78-injected joints were analyzed by measuring circumference, articular index, and by histopathology. Each rat’s four joints were measured as described, and their sum totaled. The anti-ENA-78 and control IgG animals had similar sum circumferences (181 ± 6 mm for control IgG vs 178 ± 15 mm for anti-ENA-78) (Fig. 7A). The articular index also showed no significant difference between the two groups (6.1 ± 4.3 for control IgG vs 4.8 ± 2.8 for anti-ENA-78) (Fig. 7B). A significant positive correlation was found between joint circumference and articular index ($r = 0.93; p < 0.05, n = 10$). Histologic analysis was performed on sections of the hind limb ankle joints for control and treated groups to determine the number of macrophages, lymphocytes, PMNs, and blood vessels. No significant differences between control IgG-treated ST sections and anti-ENA-78-treated sections were detected (Table III).

The reactivity of ST cells with anti-IL-1β and anti-TNF-α was determined (Figs. 8 and 9). IL-1β reactivity of ST lining cells was reduced significantly in anti-ENA-78-treated ankles compared with the control IgG ankle sections (74 ± 8.9% for control IgG vs 5.8 ± 1.8% for anti-ENA-78; $p < 0.05$) (Fig. 8A). In addition, rat IL-1β was determined in ankle homogenates by ELISA (Table II). Anti-ENA-78 treatment resulted in a slight decrease from a mean of 114.7 ± 43.8 pg/ml for IgG-treated animals to a mean of 97.6 ± 59.5 pg/ml with anti-ENA-78 Ab ($n = 7$). While mean numbers of TNF-α-immunopositive lining cells decreased with anti-ENA-78 treatment (31.8 ± 14.7 vs 13.3 ± 8.3), these results did not attain statistical significance ($p < 0.05$). Analysis of rat TNF-α by ELISA assay in ankle homogenates demonstrated a mean 10-fold decrease when anti-ENA-78 Ab was administered (Table II). Values of TNF-α in the control IgG-treated ankle homogenates dropped from a mean of 34.2 ± 16.2 pg/ml to a mean of 3.9 ± 2.1 pg/ml with anti-ENA-78 treatment.

**FIGURE 8.** Percentage of reactivity of ST cells with IL-1β or TNF-α. Animals were given adjuvant to induce AIA, then injected after the onset of arthritis with either control IgG or anti-ENA-78 on days 18, 20, and 22 postadjuvant injection, and sacrificed on day 24 postadjuvant injection. Immunohistochemistry detecting IL-1β (A) or TNF-α (B)-immunopositive cells was performed on frozen synovial sections. Stars indicate statistically different values between groups ($p < 0.05$).

**FIGURE 9.** Immunohistochemical reactivity of IL-1β with ST sections of anti-ENA-78-treated and control IgG rats after onset of AIA. Animals were given adjuvant and then injected with either control IgG or anti-ENA-78 on days 18, 20, and 22 postadjuvant injection. Frozen tissue sections of joints from anti-ENA-78-treated animal joints (A) or IgG-treated control animals (B) were examined for IL-1β immunoreactivity on day 24 postadjuvant injection ($\times 295$).

### Table III. Number of macrophages, lymphocytes, PMNs, and blood vessels per high power field for animals treated with anti-ENA-78 or control IgG after onset of AIA

<table>
<thead>
<tr>
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<th>Control IgG</th>
<th>Anti-ENA-78</th>
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<tr>
<td>No. of macrophages</td>
<td>71 ± 6</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>No. of lymphocytes</td>
<td>49 ± 3</td>
<td>83 ± 13</td>
</tr>
<tr>
<td>No. of PMNs</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>No. of blood vessels</td>
<td>9 ± 1</td>
<td>10 ± 1</td>
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* Values represent the mean ± SEM of three high power fields ($n = 3$ animals per group, 1 ankle per animal).

*Animals were given adjuvant, injected with either control IgG or anti-ENA-78 on days 18, 20, and 22 postadjuvant injection, and sacrificed on day 24 postadjuvant injection. Frozen synovial sections were examined.
Discussion

ENA-78 is a member of the human C-X-C chemokine supergene family. Like the other members of the C-X-C chemokine family that contain an ELR (Glu-Leu-Arg) motif, it is a chemotaxin for PMNs and an angiogenic factor (10, 11). Chemokines may have a role in inflammatory diseases such as chronic and acute lung disorders, which are characterized by infiltrates of PMNs in the lungs as well as increased chemotactic activity for PMNs (13, 27, 28). ENA-78 may also play an important role in chronic inflammation of the RA joint and in the recruitment of PMNs into the RA joint. ENA-78 is one of the most abundant chemokines present in human SF, being found more than 15 times higher in concentration than mean IL-8 levels (14). ENA-78 is increased in RA ST compared with normal ST and increased in SF from RA compared with osteoarthritis or other inflammatory or noninflammatory forms of arthritis (14). Therefore, the present study was conducted to characterize ENA-78-like protein expression in the development of AIA in Lewis rats, a model for RA, as well as to attempt to modify the progression of AIA with administration of neutralizing anti-ENA-78. This Ab has been used previously in rats to significantly attenuate pulmonary neutrophil sequestration and microvascular permeability in a model of hepatic ischemia/reperfusion injury (29). We demonstrate in this study that this Ab inhibits LPS-induced rat PMN recruitment to the peritoneum, further suggesting that it neutralizes an endogenous rat ENA-78-like protein that can recruit PMNs in vivo.

We found a significant increase of ENA-78-like protein levels in the serum in AIA animals compared with control animals starting at day 7 postadjuvant injection. Serum ENA-78-like protein levels continued to increase as the disease developed. This increase in expression as AIA developed was also seen for ENA-78-like protein expression in the joint homogenates. It should be noted that ENA-78-like protein was found in control and AIA animals on day 0, indicating that rats have a low-level constitutive expression of this protein in their joints. ENA-78-like protein expression in the joint as well as in the serum correlated with the increase in joint circumference of the animals as the disease developed.

Administering neutralizing anti-ENA-78 to AIA animals resulted in a decrease in the severity of arthritis. The timing of the anti-ENA-78 treatment was critical for inflammation to be affected. It was necessary to treat animals with anti-ENA-78 before the onset of disease. We have noted in a previous study (22) (data not shown) that the sum joint circumference of nonarthritic mineral oil-injected rats did not change in a 25-day period by more than 0.6%. Therefore, the initial sum joint circumference on day 0, taken before adjuvant was injected, may serve as a reference for determining how much anti-ENA-78 treatment has actually altered AIA. The initial sum joint circumference of animals used in this study was 151.2 mm. In our preventative model, as shown in Fig. 4, the sum joint circumference was reduced from a mean of 167.9 mm in control animals to a mean of 156.9 mm with anti-ENA-78 treatment, a 66% reduction of the induced swelling. Thus, anti-ENA appears beneficial when given in a preventative manner. However, when anti-ENA was given after AIA development, Fig. 7 shows that the sum joint circumference was reduced from a mean of 180.9 mm in control animals to a mean of 177.6 mm in anti-ENA-treated animals. Thus, giving the Ab after disease development only alleviated the induced swelling by 11%.

Other AIA treatment protocols have shown a similar dependency on the timing of treatment. Williams and coworkers, using an AIA model, found that when methotrexate treatment was initiated on the day of arthritis induction, the drug suppressed development of joint inflammation (30). However, when giving methotrexate on day 11 postadjuvant, no significant suppression in joint inflammation was observed (30). Conversely, Bradbeer and coworkers demonstrated treatment of AIA with the compound SK&F 106615 on either days 0–16 postadjuvant injection (prophylactically) or days 10–23 (therapeutically) resulted in significant improvement in disease for both protocols (31). These studies show that the progression and establishment of AIA depend not only on the presence or absence of certain factors, but also suggest a disease-stage specificity for their actions. Thus, our results support a role for ENA-78-like protein in the early stages of the disease and as an initial cytokine in the complex network of cytokines functioning in the inflamed joint. While it does not appear to have these effects through altering the number of cells, it is possible that the activation state of the cells may be a contributing factor to the degree of arthritis in the anti-ENA-78-treated animals. Once the disease is established, the cells in the joint are producing numerous cytokines with overlapping functions that are capable of maintaining chronic inflammation. It is likely that due to the redundancy of the cytokines, the removal of ENA-78-like protein at this stage no longer has an effect.

AIA and murine type II collagen-induced arthritis (CIA) demonstrate the complicated interactions of cytokines and chemokines involved in the arthritic process. Issekutz and coworkers investigated the role of endogenous IL-1α, IL-1β, and TNF-α in the leukocyte recruitment and the inflammatory responses in the joints of AIA rats (18). They observed that anti-TNF-α Ab treatment significantly improved clinical score, local inflammation, and the infiltration of PMNs, and to a lesser extent T cells, into the joint. In contrast, anti-IL-1α or anti-IL-1β treatment alone was ineffective, although when combined with anti-TNF-α, a significant effect on T cell migration was observed. Thorbecke and coworkers showed that induction of CIA in DBA/1 mice was reduced significantly if TGF-β or anti-TNF-α were administered systemically, whereas anti-TGF-β or TNF-α increased the incidence of CIA (32). Kasama and coworkers analyzed the expression of IL-10 and the chemokines MIP-1α and MIP-2 and their contribution to the evolution of CIA (33). They found anti-IL-10 treatment increased the expression of MIP-1α and MIP-2, as well as myeloperoxidase activity and leukocyte infiltration in the inflamed joints. These studies as well as ours demonstrate the complexity of the cellular interactions within the AIA synovium.

ENA-78-like protein may be involved in the progression of AIA in concert with other cytokines. Expression of ENA-78-like protein in the joint during the progression of AIA correlated with the expression of several proinflammatory cytokines, namely TNF-α, IL-6, IL-1, and MIP-1α (manuscript in preparation). Furthermore, ENA-78-like protein expression is inducible by both the proinflammatory cytokines, TNF-α and IL-1β. When the human type II epithelial cell line, A549, was stimulated with either TNF-α or IL-1β, ENA-78 release was increased, as was the release of several other chemokines (13). RA synovial tissue fibroblasts stimulated with TNF-α in vitro increased their secretion of ENA-78 (14). In addition, human ENA-78 is an ELR-containing CXC chemokine that is chemotactic for endothelial cells as well as angiogenic in vivo (34). These are biological functions that may be shared with the ENA-78-like protein that is recognized by anti-ENA-78.

A sequential rather than simultaneous expression of TNF-α and IL-1β, with TNF-α being expressed first, has been suggested for CIA development in mice (35, 36). Marinova-Mutafchieva and coworkers found progressive increases in the number of TNF-α- and IL-1β-positive cells in the joints of CIA mice as arthritis developed (35). They also reported TNF-α expressed in the joints early in the study (day 1), while IL-1β was not expressed in the joints until day 3 (35). Furthermore, Joosten and coworkers found
anti-TNF-α treatment of CIA to be effective only if administered early after onset of CIA and not in late stages of CIA, whereas anti-IL-1β was effective when administered early or late in CIA (36).

It is possible that ENA-78 may also modulate the expression of IL-1β and/or TNF-α. To address this possibility, we performed ELISAs and immunohistochemistry on joint tissue. When anti-ENA-78 was administered before the onset of CIA, mean IL-1β levels decrease 73%, as determined by ELISA. In agreement with this finding, the percentage of IL-1β-immunopositive lining cells decreases, which most likely contributes to the overall decrease, as demonstrated by ELISA. In contrast, anti-ENA-78 Ab treatment before CIA onset did not decrease TNF-α levels in ankle homogenates, as determined by ELISA, although the mean percentage of immunopositive lining cells decreased. TNF-α in joint homogenates, which include cells extract from cartilage and bone, may show TNF-α levels reflective of these other nonsynovial cell types in the joint milieu, thus accounting for the disparate immunohistological and ELISA results.

When anti-ENA-78 was administered after the development of CIA, ELISA data demonstrate that IL-1β levels decrease slightly (15%). This decrease was most likely supported by a decrease of IL-1β produced by synovial lining cells, since the percentage of immunopositive IL-1β lining cells was significantly decreased. Furthermore, anti-ENA-78 treatment after CIA onset decreased the quantity of TNF-α by 89%, as determined by ELISA. Immunohistochemical data suggest that lining cells, but not sublining macrophages, may have played a role in this overall reduction in TNF-α. These results suggest that a cytokine network involving IL-1β, TNF-α, and ENA-78-like protein is present in the AIA joint.

In rat peritoneal inflammation, anti-ENA-78 clearly reduces the PMN influx in response to rENA-78 or LPS. In rat CIA, however, treatment with anti-ENA-78 Ab before CIA onset decreased the mean number of PMNs by 85%, as determined histologically in the joint, but this decrease was not statistically significant. A number of possible explanations could account for this result. These include: 1) imperfect cross-reactivity and/or neutralization of the rat ENA-78-like protein with the anti-human ENA-78-like protein; 2) ineffective injection times, dosage, and/or method of administration (i.e., injection) of the Ab for an efficacious neutralization of the ENA-78-like protein; and 3) variability in the data with a large SE accounting for lack of statistical significance. It is also possible that different homing pathways exist for PMNs to be recruited into the joint versus the peritoneum. In both rats and humans, distinct organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). Hence, PMNs may respond to organ-specific homing mechanisms have been identified for inflammatory leukocytes (37–39). 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