Cationic Yersinia antigen-induced chronic allergic arthritis in rats. A model for reactive arthritis in humans.

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Research Article

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Cationic *Yersinia* Antigen-induced Chronic Allergic Arthritis in Rats

A Model for Reactive Arthritis in Humans

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Abstract

Cationic antigens are known to have considerable arthritogenic potential in experimental systems. During a systematic search for suitable, naturally occurring candidates an intracellular protein was isolated from the ribosomal pellet of *Yersinia enterocolitica* 0:3, a bacterial strain associated with reactive arthritis in humans. The protein is highly cationic, contains two 19-kD polypeptide chains linked by a disulfide bond, and reveals a strong tendency for spontaneous aggregation. It is suggested to be a nucleic acid binding protein. We tested this antigen for its ability to induce arthritis after intra-articular challenge in preimmunized rats. An acute inflammatory phase followed by transition to chronicity was observed both by technetium-99m scintigraphy and from histology. Massive polymorphonuclear leucocyte infiltration of the synovium was seen early on and fibrosis and thickening of the joint capsule occurred in later stages. Control groups showed no evidence of inflammation. Western blot and ELISA analysis of unselected sera from *Yersinia enterocolitica* 0:3–infected patients revealed antibodies to the antigen in the majority of cases, whereas healthy individuals rarely reacted. This is the first report of a naturally occurring cationic antigen capable of inducing immunologic tissue injury; it justifies the speculation that cationic antigens from prokaryotic cells could trigger reactive arthritis in humans. (*J. Clin. Invest.* 1991. 87:632–642.)
Key words: *Yersinia* • arthritis • cationic antigen

Introduction

Reactive arthritis is a relatively common complication in individuals suffering from enteric infections with *Yersinia enterocolitica* (Y.e).[1] It develops typically 1–4 wk after onset of enteritic symptoms and the clinical course varies between acute, self-limiting forms in the vast majority of cases, as well as intermittent relapsing and finally chronic forms in up to 10% of those affected (1); about 10% of patients with arthritis develop Reiter's syndrome.

Reactive arthritis is defined by its association with a recognized microbial infection; in the past there was a general consensus regarding the absence of viable microbes or microbial products in the affected joint. In the case of *Yersinia enterocolitica* this opinion has been substantially modified by the investigations of Toivanen, Lahesmaa-Rantala, Granfors, and coworkers in Finland. These workers could demonstrate the presence of *Yersinia* antigens in both immune complexes (2) and cells (3) from the synovial fluid of patients suffering from reactive arthritis, and moreover probably as deposits in the patient's synovium itself (4). These findings are analogous to those of other investigators in Reiter's syndrome concerning deposition of chlamydial antigens, chlamydial elementary bodies, and immunoglobulin in synovial tissue or their presence in synovial fluid (5–7). On the basis of these observations in humans, one can propose an involvement of microbial antigens in the pathogenesis of this disease group.

Antigen-induced arthritis has a sound experimental basis in animals. Chronic arthritis can be induced by intra-articular administration of antigens into preimmunized animals; this system was first described in rabbits (8, 9) and later in mice (10, 11). Antigen retention by immune complex formation within collagenous tissues of the joint was proposed as the major mechanism for induction and maintenance of this disease in rabbits (12, 13). In contrast, quite different conditions exist in mice. Native proteins like BSA, useful as an arthritogen in rabbits, lacked any arthritogenic potential in rodents. In further contrast to this, molecules rendered strongly cationic by chemical modification were recognized to be powerful arthritogens due to their electrostatic trapping by polyanionic joint structures (14).

Besides the influence of charge, size was shown to be another important determinant for arthritogenicity of antigens in the mouse model (15, 16). Similar results were obtained in an analogous rat model (Gondolf et al., submitted for publication).

The purpose of the present work was to develop an animal model for human reactive (postinfectious) arthritis using cationic, native bacterial components as antigen and exploiting conclusions from earlier studies with artificially altered proteins. During the search for suitable candidates for arthritis induction we identified a cationic intracellular, putative nucleic-acid binding protein from *Yersinia enterocolitica* 0:3, which was a potent arthritogen in rats.

Methods

Animals

Male Wistar rats (Zentraltierzüchterei Hannover, Hannover, FRG) were used throughout. The body weight was 100 g at the beginning of the experiments. Intra-articular injections were carried out under ether narcosis.

Preparation of antigen

A Y.e. 0:3 strain, isolated from a patient suffering from enterocolitis, was grown in 21 vol of brain-heart infusion medium (Oxoid Ltd., Ba-

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1. *Abbreviation used in this paper:* Y.e., *Yersinia enterolitica.*

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singstroke, Hants., England) at 30°C and pH 7.0 in a fermenter (Applikon, Schiedam, The Netherlands). 10% glucose solution was added at regular intervals. After reaching the stationary phase, cells were harvested by centrifugation (2,000 *g*, 15 min) (Sorvall model RC-5B, DuPont Co., Bad Hoburg, FRG) and the wet weight was estimated. The two cultures (50 g) were resuspended in 10 mM Tris-HCl, pH 8.0, HCl, pH 8.0, containing 100 mM $MgCl_2$ and 1 mM PMSF (2 ml/g wet weight) and disrupted in a homogenizer (Braun, Melsungen, FRG) using glass beads with a diameter of 0.1–0.11 mm. After extensive washing in the above-mentioned buffer the glass beads were removed by sedimentation. Further procedures were carried out as described by Lathe and co-workers (17) with certain modifications. Briefly, the remaining supernatant was incubated at 37°C for 1 h in the presence of DNAase I (Sigma Chemical Co., Deisenhofen, FRG) at a concentration of 10 μg/ml. Thereafter intact cells were removed by centrifugation (2,000 *g*, 20 min) and the supernatant was centrifuged twice at 37,000 *g* for 1 h (sediment called membrane pellet) and then at 150,000 *g* for 2 h (4°C). The final sediment, the ribosomal pellet (150,000 *g*) was extracted with 66% acetic acid containing 33 mM Mg acetate under continuous stirring overnight, followed by ultracentrifugation at 200,000 *g* for 2 h to remove nucleic acids. Proteins were precipitated by adding 5 vol of acetone, dried under vacuum, and redissolved in 0.1 N HCl. Insoluble material was again removed by ultracentrifugation at 200,000 *g* for 2 h. The resulting supernatant was transferred to PBS by stepwise dialysis. The first dialysis was made against 0.01 N HCl, and the pH and ionic strength were then slowly raised by repeated replacement of one quarter of dialysis solution by PBS. The final sample (crude ribosomal pellet extract) was frozen and stored at −20°C until further use.

The 150,000-*g* supernatant was recentrifuged to remove residual ribosomes and incubated at 100°C for 1 h. The precipitate was removed by centrifugation (10,000 *g*, 15 min), and 37% HCl was added to the supernatant to a final concentration of 0.25 N. Insoluble material was again removed by centrifugation and the soluble proteins were precipitated by adding 5 vol of acetone. The proteins were resolubilized with 0.1 N HCl and stored at 4°C until examination.

The proteins of the membrane pellet (see above) were extracted with 0.25 N HCl under continuous stirring at 4°C overnight; insoluble material, including nucleic acids, was removed by ultracentrifugation (200,000 *g* for 2 h); and dialysis against PBS was performed as described above.

The extracted fraction of the ribosomal pellet contained the protein of interest (see Results) and was further separated either by repeated size-exclusion chromatography or by ion-exchange chromatography followed by size exclusion chromatography (see Results).

Size-exclusion chromatography

A Sephadex G-100-superfine column (Pharmacia, Freiburg, FRG) of 2.5 × 90 cm in PBS buffer at 4°C was used. The column was calibrated for molecular mass estimation using horse spleen ferritin (480 kD, prepared according to Vogt et al. [18]), BSA (68 kD, Behring, Marburg, FRG), streptococcal proteinase (30 kD, prepared according to Elliott and Liu [19]), and lysozyme (14-kD, Sigma Chemical Co.). A constant flow rate of 10 ml/h was maintained and the eluate was monitored at 280 nm.

The column was then loaded with 10–25 mg of the protein fraction (crude ribosomal pellet extract; see above). Fractions of 6 ml were collected, and the main peaks were pooled and concentrated in an ultrafiltration cell (YM10 membrane, Amicon Corp., Witten, FRG), and analyzed by SDS-PAGE.

Ion-exchange chromatography

The whole procedure was performed using a fast protein liquid chromatography system (Pharmacia). After equilibration with 0.15 M NaCl in 10 mM phosphate, pH 7.4 (starting buffer), a Mono S-cation exchange column, HR 5/5 (Pharmacia), was loaded with 20 mg of the sample in starting buffer. Elution was performed with a sodium chloride gradient ranging from 0.15 to 2 M, followed by a single application (3 ml) of 6 M guanidine, pH 7.4, with the flow rate 0.5 ml/min. 1-ml fractions were collected and analyzed by SDS-PAGE before they were pooled.

Determination of protein concentration

The protein concentration was measured by bicinchoninic acid protein assay reagent (20) (Pierce Europe, Oud Beijeland, The Netherlands) with BSA as standard.

SDS-PAGE

The protein samples were analyzed by SDS-PAGE according to Laemmli (21) under reducing and nonreducing conditions, without boiling (gel concentration T = 15%, C = 2.7%). For molecular mass determination a standard protein mixture (range 14–94 kD, Pharmacia) was used. The gels were stained with Coomassie Blue R 250. For autoradiography of ^{125}I-labeled proteins the gels were fixed in 40% (vol/vol) ethanol and 10% (vol/vol) acetic acid, dried, and then exposed to an X-ray film (Eastman Kodak Co., Rochester, NY) using titan enhancer plates (Siemens, Freiburg, FRG).

Isoelectric point determination

The isoelectric point (pI) was estimated by isoelectric focusing in polyacrylamide gels (T = 5.5%, C = 2.7%) containing 6% ampholyte (pH range 3.5–10; Pharmacia), using pI markers (pI range 3–10, Pharmacia).

Radioisotopic labeling of proteins

Lysozyme, histone f1 (22) and *Yersinia* antigen (see Results) were labeled with ^{125}I (Amersham, Branschweig, FRG) by the chloramine-T method (23). Free ^{125}I was removed by exhaustive dialysis against PBS. Before use, the ^{125}I-labeled proteins were mixed with nonlabeled protein.

Affinity to polyanionic structures in vitro

Heparin-Sepharose chromatography. Affinity chromatography was performed using a 1.0 × 0.5-cm column containing 200 μl of packed heparin-Sepharose CL-6B (Pharmacia). 50 μg of ^{125}I-labeled samples (1.5×10^6 cpm) was applied in a total volume of 100 μl, and an NaCl gradient of 0.15–2 M, in 10 mM sodium phosphate, pH 7.4, was run at a flow rate of 0.2 ml/min. Final elution was made with 6 M guanidine-HCl, pH 7.4. 500-μl fractions were collected and the radioactivity in each fraction was measured in a gamma counter (Berthold, Wildbad, FRG).

DNA-cellulose chromatography. Affinity for DNA was studied using a 0.8 × 0.5-cm column containing 150 μl of packed DNA-cellulose (Pharmacia) equilibrated in a low-salt starting buffer containing 20 mM Tris-HCl, 10 mM EDTA, 10% glycerol, and 50 mM NaCl, pH 8.0. After washing, the column was loaded with 20 μg of ^{125}I-labeled samples (1.5×10^6 cpm) in a total volume of 100 μl of starting buffer. Elution was done with an NaCl gradient of 0.15–1 M, final elution was done with 6 M guanidine-HCl, pH 8.0 using a constant flow rate of 0.2 ml/min, 500-μl fractions were collected, and the radioactivity was measured.

Antiserum

Antisera were raised in rabbits. Immunization was performed subcutaneously four times at monthly intervals with 250 μg of *Yersinia* antigen (see Results, Fig. 1, peak 1*b*), emulsified in complete Freund's adjuvant. Serum was taken 2 wks after the last injection and tested in a gel diffusion test as well as by Western blot and ELISA. The specificity was confirmed by absorption: 200 μl of antiserum was incubated with 70 μl of highly purified *Yersinia* antigen (see above) at 37°C for 1 h. A flocular precipitate formed, which was removed by centrifugation, and the supernatant was tested.

Studies on other bacteria

Two isolates (stool) of *Escherischia coli, Salmonella enteritides, Shigella sonnei,* and *Campylobacter jejuni* as well as two Y.e. 0:9 strains

and two other Y.e. serovars were cultured, disrupted by ultrasonication, and subjected to SDS-PAGE followed by Western blotting with specific anti-Y.e. 0:3 19-kD antiserum.

Human sera

Samples of human sera were taken from patients with Y.e. caused enteric disease. The diagnosis was based on the presence of raised agglutinin titers (> 1:80) to boiled and formalized antigens, together with isolation of Y.e. from the faeces, in all cases. A collective of 49 patients was studied. Y.e. strains isolated were Y.e. serotypes 0:3 (n = 35), 0:9 (n = 3), 0:3 + 0:9 (n = 1), 0:6.30, (n = 1), biovar 1, (n = 4), biovar 3 (n = 3), biovar 2 (n = 1), and *Y. frederiksenii* (n = 1). The control collective (n = 31) consisted of sera taken from pregnant women during routine screening.

Western blot procedure

Yersinia antigen (see Results) was run on an SDS-PAGE gel (T = 15%, C = 2.7%) under mild reducing conditions (0.5% β-mercaptoethanol; see Results). The proteins were electrotransferred (24) to Immobilon-P membranes (Millipore Corp., Bedford, MA). Membrane strips were incubated overnight with patient's sera diluted 1:100, followed by incubation with 1:5,000 diluted peroxidase-labeled goat anti-human IgG (Dianova, Hamburg, FRG) for 1 h. Finally, the strips were developed with diaminobenzidine (Sigma Chemical Co.).

ELISA

Antibody levels were measured using an ELISA system. Wells of flat-bottomed microtiterplates (Greiner, Nürtingen, FRG) were coated with 0.05 μg of *Yersinia* antigen (see Results) or BSA. Incubation with the chosen dilution of a serum sample and 1:5,000 diluted peroxidase-labeled goat anti-human (or rat) IgG was performed. After development the OD_{492nm} was measured in a Titertek Multiscan MMC (Flow Laboratories, Meckenheim, FRG).

Skin testing

Delayed-type hypersensitivity was measured 48 h after injection of 5 μg of antigen in 5 μl PBS into the pinna of the ear. The contralateral ear, injected with PBS, served as control. Increase in ear thickness was measured with an engineer's micrometer.

Histology

Knee joints were detached and fixed in 4% buffered formalin, decalcified, and embedded in paraffin. Standard frontal sections of the joint were cut and stained with hematoxilin and eosin.

Technetium-99m uptake measurements

Joint inflammation was determined by ^{99m}Tc pertechnetate uptake measurements of the knee joints; this method correlates well with histologic scores in arthritic joints (25, 26). Each rat was injected with 2 MBq ^{99m}Tc in 0.1 ml of PBS subcutaneously into the neck region; 30 min later the intensity of gamma radiation over both knee joints was measured by a collimated photoscintillation crystal (Berthold, Wildbad, FRG), keeping the knee joints in a defined position and carefully shielding the bodies of the rats. Inflammation was scored as the ratio of the ^{99m}Tc uptake in the left vs. that of the right knee joint (L/R ratio).

Antigen retention in vivo

25 μg of ^{125}I-labeled samples were injected in 50 μl of PBS into the left knee joint of rats; the right knee joint received PBS. At various days thereafter radioactivity was measured over both knees by external gamma counting, similar to the ^{99m}Tc uptake measurements (see above). Values from the left knee were corrected by deducting counts in the right knee, which resulted from blood contamination. Three groups of nonimmune rats (n = 4), injected with either lysozyme, histone f1, or *Yersinia* antigen (see Results) were compared. The studies in nonimmune rats were performed to obtain data on the charge-mediated binding of three different polycationic molecules in vivo. Another group of rats (n = 4) immunized with *Yersinia* antigen was studied to examine the additional influence of antibody-mediated retention.

Immunization and intra-articular injection

Rats were immunized with 100 μg of antigen, emulsified in complete Freund's adjuvant (total volume 300 μl) by subcutaneous injection into the dorsal region, and boostered twice with the same dose at 2-wk intervals, until an adequate immune response was induced. Adjuvant was not injected into the hindleg region to avoid joint interference. Intra-articular injections were carried out using a 27-gauge needle inserted into the hindleg knee joint space; the standard injection volume was 50 μl.

Arthritis induction

Four groups of immunized rats were challenged in the left knee joint with 50 μg of the appropriate antigen (see Table I and Results). In group 1 (arthritic), joint inflammation was determined by ^{99m}Tc uptake measurements at days 2, 6, 14, 24, 34, and 44; left and right knee joints from two rats were taken for histological examination at days 2, 6, 26, 40, and 78. In the control groups (2, 3) ^{99m}Tc uptake was measured at days 2 and 10, and histological examination was performed on knee joints from two rats per group at day 6. Preparation of the tetrameric lysozyme used in group 2, as well as the control group 4, designed to show that polyanionic antigens are not arthritogenic in this system, has been reported in detail elsewhere (Gondolf et al., submitted for publication).

Tissue autoradiography

Dry decalcified, deparafinized tissue sections were covered by photographic emulsion (NTB 2, Eastman Kodak Co.), melted at 42°C for 30 min before use. The slides were dried for 1 h and then exposed for 14–28 d at 4°C. After exposure slides were processed in Kodak D19 developer for 150 s and the reaction was stopped by 2% acetic acid within 15 s, followed by fixation in Rapid Fix (Agfa Gevaert 1:8; Leverkusen). Finally, the slides were stained with hematoxilin and eosin.

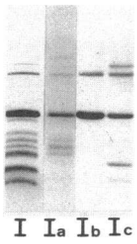
Statistical analysis

Independent groups were compared by the t test.

Results

Characterization of isolated Yersinia antigen

The ribosomal pellet derived from homogenized Y.e. 0:3 cells was composed of several proteins with a molecular mass ranging between 9 and 100 kD in SDS-PAGE; under reducing conditions, one strong band running at 19 kD (designated Y.e. 0:3 19 kD) was dominant. It eluted mainly in the major peak obtained from a first chromatography of the crude ribosomal pellet extract on a Sephadex G-100 superfine column (peak I in Fig. 1). Under nonreducing conditions a 38-kD band in SDS-PAGE appeared, which in fact could be converted into the 19-kD band by 5% mercaptoethanol (Fig. 2 *b*); we appear to be dealing with a thiol-stabilized dimer. Using much lower concentrations of mercaptoethanol (0.5%), both the 38- and the 19-kD forms were visible (these conditions were used in the Western blot procedure for testing patients' sera; see Fig. 3). The material found in peak I appears to represent the dimer protein complexed with other molecules. Further purification could be achieved by a second run of peak I under the same conditions, three main peaks resulted (Fig. 1). Each peak contained the protein of interest (Y.e. 0:3 19 kD) in varying amounts, the highest quantity appeared in peak I*b* (for protein content see also Fig. 2 *a*, lane *1*), whose elution volume was close to that of BSA (68-kD), which indicates a degree of aggregation. Peak I*a*, the exclusion fraction, represents a highly ag-



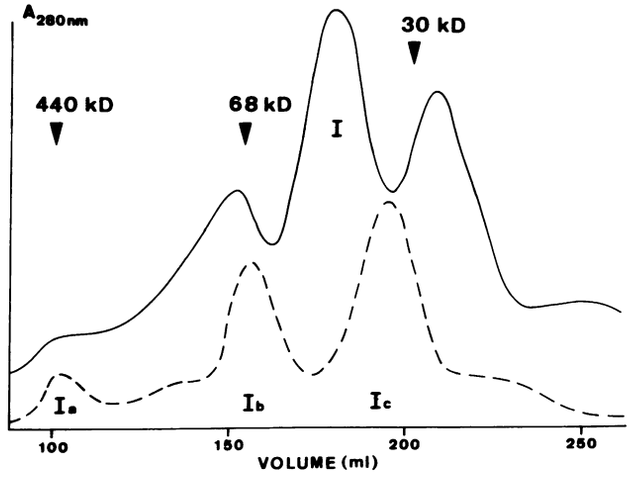


Figure 1. Repeated purification of the crude ribosomal pellet extract by gel filtration (Sephadex G-100 superfine). The major peak of the first run (peak I), containing the Y.e. 0:3 19 kD, was rechromatographed; peak I*b* running as 68 kD consisted preferentially of Y.e. 0:3 19 kD.

gregated form of the protein. Peak I*c* contained small-sized proteins in a high concentration, but also the Y.e. 0:3 19-kD protein in dimeric but nonaggregated form.

When ion-exchange chromatography was chosen as the first purification step of the crude ribosomal pellet extract, considerable amounts of Y:e 0:3 19-kD protein in dimeric and aggregated form could only be found in the large peak eluting with 6 M guanidine (Fig. 2 *a*, lane *3* reveals protein content, elution profile not shown). A low proportion of Y.e. 0:3 19-kD in highly purified form was present in a peak eluting with 0.3 M NaCl (probably representing the nonaggregated form), traces were also found throughout the whole salt gradient, together with other proteins (demonstrating a strong tendency for aggregation with other proteins). When the peak eluting with 6 M guanidine in ion-exchange chromatography was subsequently run on a Sephadex G-100 Superfine column, a profile similar to that of the second run shown in Fig. 1 was seen (not shown).

By isoelectric focusing the 19-kD protein revealed a pI $\geq$ 9.3. Exact determination was not possible since the protein migrated into the cathodal strip (Fig. 2 *c*). Only traces of the 19-kD protein could be found in the membrane fraction (37,000 *g*) (not shown), whereas in the 150,000 *g* supernatant the 19-kD protein was also present (not shown), but in quantities far lower than in the ribosomal pellet.

Prevalence of the 19-kD protein in other bacteria

The four other Y.e. (non 0:3) strains tested also contained the 19-kD protein, but in much lower amounts than seen in the 0:3 serovar. The other enterobacteria tested (*E. coli, S. enteritides, S. sonnei,* and *C. jejuni*) were all negative.

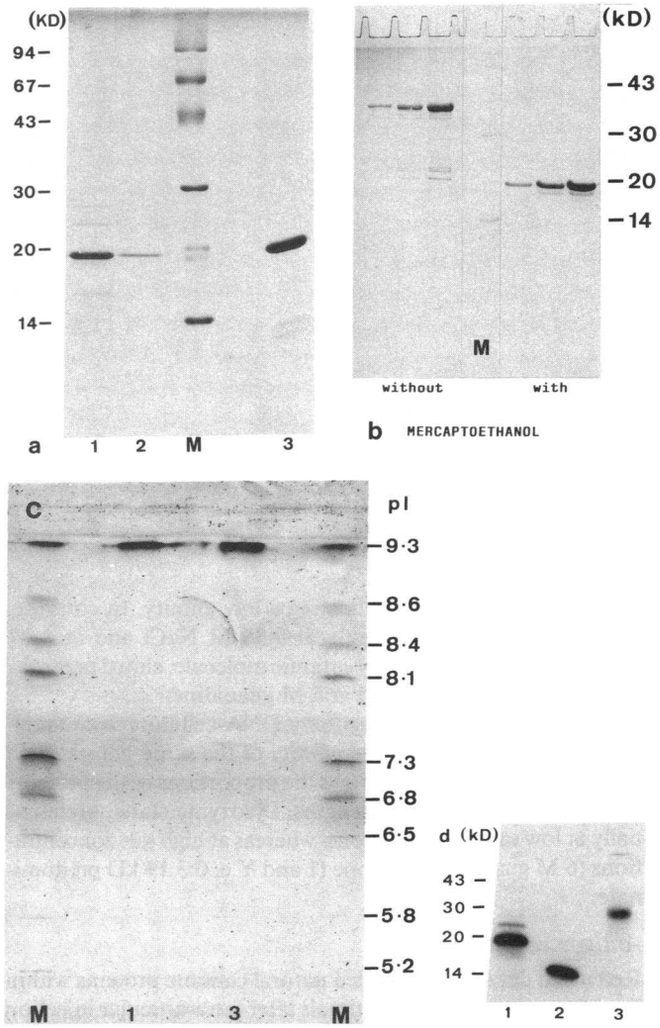


Figure 2. (*a*) SDS-PAGE (T = 15%, C = 2.7%) under reducing conditions showing different Y.e. 0:3 19-kD preparations: lanes *1* and *2*, content of peak I*b* from gel filtration (Fig. 1); lane *3*, content of the peak eluting with 6 M guanidine from Mono S cation exchanger. Major band is the Y.e. 0:3 19-kD protein. *M*, molecular weight markers. (*b*) SDS-PAGE under nonreducing (*left*) and reducing conditions (*right*). Under nonreducing conditions the Y.e. 0:3 19 kD appears as a 38-kD band, apparently a disulfide bond–linked dimer of the 19-kD protein. *M*, molecular weight markers. (*c*) IEF in polyacrylamide gel (pI range 5–9.3) showing different Y.e. 0:3 19-kD preparations. Lanes *1* and *3* are the same samples as in *a*, lanes *1* and *3*. (*d*) SDS-PAGE under reducing conditions followed by autoradiography. Lane *1*, ^{125}I-Y.e. 0:3 19 kD; lane *2*, ^{125}I-lysozyme; lane *3*, ^{125}I-histone f1. The three proteins used for heparin-Sepharose chromatography, DNA-cellulose chromatography, and antigen retention studies in vivo are proportionally radiolabeled.

Affinity for polyanionic structures in vitro

For these studies ^{125}I-labeled preparations were used (see Fig. 2 *d*); the Y.e. 0:3 19-kD preparation used was aggregated and corresponded to fraction I*b* (Fig. 1).

Heparin-Sepharose chromatography. A heparin-Sepharose column was used to compare the binding of Y.e. 0:3 19 kD, histone f1, and lysozyme to a chondroitin sulfate–like matrix. The data are shown in Fig. 4. Except for a small peak with 6 M guanidine, lysozyme elutes almost entirely at low salt concen-

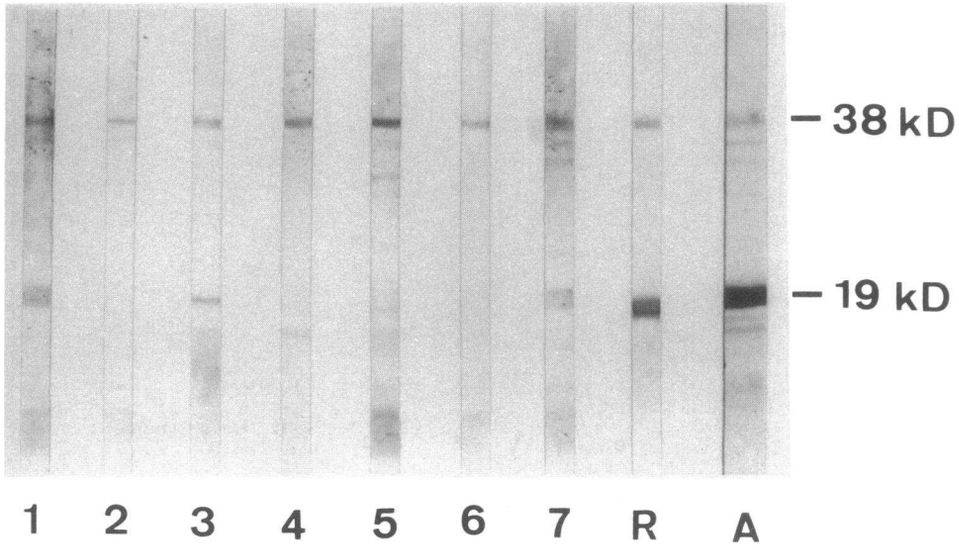


Figure 3. Western blot of sera from patients with proven Y.e. 0:3 enteritis (strips *1–7*). *R*, specific rabbit antiserum against Y.e. 0:3 19-kD antigen. *A*, amido black stain of the Y.e. 0:3 19-kD protein. Patients sera preferentially react with Y.e. 0:3 19-kD dimer. For the SDS-PAGE mild reducing conditions were selected to achieve only partial conversion of the dimeric antigen into its 19-kD monomer.

trations (0.15 M NaCl), indicating a low affinity. In contrast, Y.e. 0:3 19 kD eluted mainly at 0.36 M NaCl and at 6 M guanidine. Histone f1, a very cationic molecule, eluted predominantly at 0.84 M NaCl and at 6 M guanidine.

DNA-cellulose chromatography. DNA-cellulose chromatography was used to study the affinity of the same polycationic molecules to DNA. Fig. 5 shows the proportions of the proteins eluting at different ionic strengths. Lysozyme eluted preferentially at low salt concentrations, whereas at high salt concentrations (6 M guanidine), histone f1 and Y.e. 0:3 19 kD predominate.

Antigen retention in vivo

Retention data for ^{125}I-labeled natural cationic proteins within the joints of nonimmune animals after intra-articular injection are shown in Fig. 6 *a*. Lysozyme disappeared most rapidly from the joint. The persistence of Y.e. 0:3 19 kD (aggregated; see fraction I*b*, Fig. 1) and histone f1 was prolonged to a comparable extent. This is in accordance with the results of affinity studies to polyanionic matrices in vitro (see above and Figs. 4 and 5). In immune rats retention of Y.e. 0:3 19 kD showed a further significant increase (Fig. 6 *b*).

Distribution of Y.e. 0:3 19-kD in the joint

Autoradiography was performed 6 h after injection of ^{125}I-labeled Y.e. 0:3 19 kD and showed the antigen to be distributed in the synovial tissue as well as on the surface and in the deep layers of the cartilage (Fig. 7).

Arthritis induction with Y.e. 0:3 19 kD

Challenge with 50 μg of Y.e. 0:3 19 kD (aggregated; see fraction I*b*, Fig. 1) into the left hindleg knee-joint of rats previously immunized with this antigen (group 1 in Table I) produced chronic arthritis; this could still be observed 78 d after injection of the antigen (Fig. 8 *d*). The histologic findings were noteworthy: in the acute phase (day 2) massive edema of the synovial lining, accompanied by a diffuse inflammatory infiltrate was seen (Fig. 8 *a*). Neutrophilic granulocytes predominated, and some histiocytes and lymphocytes were also present. The surfaces of the joint structures were partially covered by a fibrin layer. The cartilage and bone were not eroded. At day 6 arthritis was progressing to a subacute stage, with development of pannus at the juxtaposition of cartilage and synovial lining, in both the medial and lateral joint space, in severe cases (Fig. 8 *b*). Destruction of the cartilage structures was beginning. The chronic stage was reached by day 26, when sclerosis with marked increase of fibroblasts could be observed within the joint capsule. Some patchy infiltrates consisting of lymphocytes were still present (Fig. 8 *c*). At that time, mean values of the L/R ratio by ^{99m}Tc-scintigraphy were 1.1 on average after a summit of 1.35 at day 2 (Fig. 9). In more severe cases, the cartilage and synovial lining were also replaced by scar tissue. Moreover, a slight proliferation of synovial lining cells was visible. These tissue changes still persisted at day 78 (Fig. 8 *d*). At the time of antigen challenge, the antibody response of the rats to the fraction used for immunization in this group (I*b*, Fig. 1) was examined by Western blotting. All rats showed a very strong response to the 19-kD protein, although antibody to the other components of the aggregated antigen (fraction I*b*) was virtually absent. In the control groups 2, 3, and 4 (Table I), no inflammation could be observed either by ^{99m}Tc-scintigraphy (Fig. 9) or by histology (not shown).

In a further experiment three rats were immunized with Y.e. 0:3 19 kD and challenged with nonaggregated Y.e. 0:3 19-kD antigen (fraction I*c*; see Fig. 1); only a very mild, transient inflammatory reaction was seen. This latter result is not reported in detail, but underlines the importance of antigen size, in this case the degree of aggregation, for arthritogenicity.

Human sera

The serologic relevance of the Y.e. 0:3 19-kD protein in patients suffering from yersiniosis could be shown using the Western blot and ELISA procedure. In Western blot, sera of the collective of patients (n = 35) with proven Y.e. 0:3 infections clearly reacted with the Y.e. 0:3 19-kD protein in almost all cases, preferentially with the dimer (38 kD); selected sera are shown in Fig. 3. In contrast, sera of the control collective did not give a positive reaction in the majority of cases. Sera of patients infected with *Yersinia* serotypes other than 0:3 produced very faint bands at 38 kD in 50% of cases. For ELISA analysis, the OD_{492nm} of the control sera was measured at a defined dilution and the mean+3 SD was chosen as the cutoff point for a positive seroreaction in the patients; on this basis

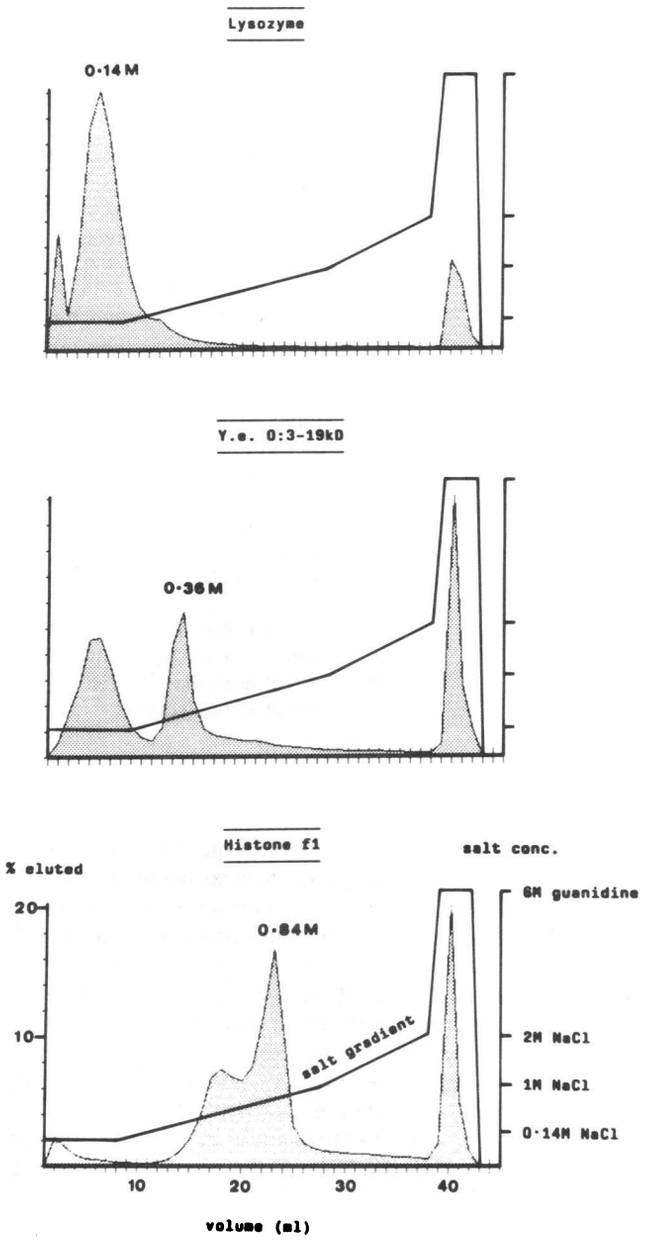


Figure 4. Elution profile of different ^{125}I-labeled cationic proteins (Fig. 1 *d*) from heparin-Sepharose. Lysozyme elutes mainly at physiologic molarity and exhibits low affinity; in contrast, most Y.e. 0:3 19 kD elutes at a higher molarity. Histone f1 has the strongest affinity to heparin-Sepharose. The peak eluting with 6 M guanidine represents a very high-affinity protein subfraction.

43% (15/35) of patients with proven Y.e. 0:3 infections were found to have a positive IgG response, whereas none of the patients infected with *Yersinia* types different from serotype 0:3 reacted positively.

Discussion

The inflammatory potential of cationic protein antigens deposited in tissues containing fixed negative charges is well established. Originally studies in rats focused attention on the pathogenesis of in situ immune complex glomerulonephritis initiated by cationic proteins (27, 28). More recently, a key role for histones, a very highly positively charged group of DNA-stabilizing molecules, in the pathogenesis of lupus nephritis was made plausible (22); this latter report directed attention upon nucleic acid binding proteins. Secondly, charge aspects have assumed relevance in the area of inflammatory joint diseases; the cartilage (29) and the synovial lining (30) possess fixed negative charges (proteoglycans) which can act as binding sites for polycationic antigens. Van den Berg and co-workers defined the charge characteristics of several proteins in relation to their ability to induce chronic allergic arthritis in mice. In contrast to anionic or neutral proteins, only highly cationized proteins could act as arthritogens in rodents (14–16). Until now, virtually all studies concerned with cationic proteins and arthritis were performed using chemically altered, i.e., cationized molecules.

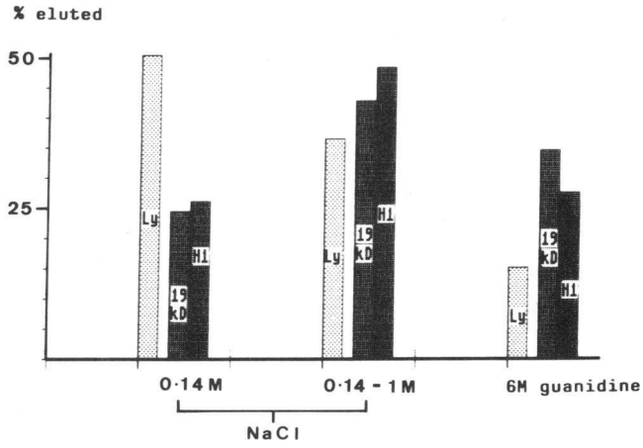


Figure 5. Elution patterns of ^{125}I-lysozyme (Ly), ^{125}I-Y.e. 0:3 19 kD (19 kD) and ^{125}I-histone f1 (H1) on a DNA-cellulose column. A salt gradient of 0.15–1 M NaCl followed by final elution with 6 M guanidine was used. Histone f1 and Y.e. 0:3 19 kD behave similarly, both show markedly higher affinity for DNA than the polycation lysozyme.

The data presented here clearly show that the cationic moiety isolated from a *Yersinia enterocolitica* strain (designated Y.e. 0:3 19-kD antigen) is a potent arthritogen in rats. The arthritis induced by the Y.e. 0:3 19-kD protein begins with an acute phase consisting of neutrophilic granulocyte infiltration of the synovium, together with edema and fibrin layer formation. This is followed by a subacute stage with formation of pannus and a chronic stage with massive sclerosis of the synovial lining, accompanied by an increase of synovial fibroblasts. Severe cases revealed spreading of inflammation to the cartilage, especially in juxtaposition to the inflamed synovium. These histopathological features closely resemble those seen in reactive arthritis in humans, where the clinical picture is also dominated by acute, patchy, nonspecific changes with infiltration by granulocytes combined with little or no synovial lining cell hyperplasia (1). In long-standing cases the experimental disease exhibits increasing resemblance to changes of rheumatoid disease, where the focus of cartilage and bone destruction occurs in juxtaposition to the inflamed synovium.

Antigen retention is an important determinant of the severity of injury in antigen-induced allergic arthritis and was given close attention in this study. When injected into the joint of nonimmune rats, the Y.e. 0:3 19-kD protein was retained significantly better than another cationic molecule, native lysozyme.

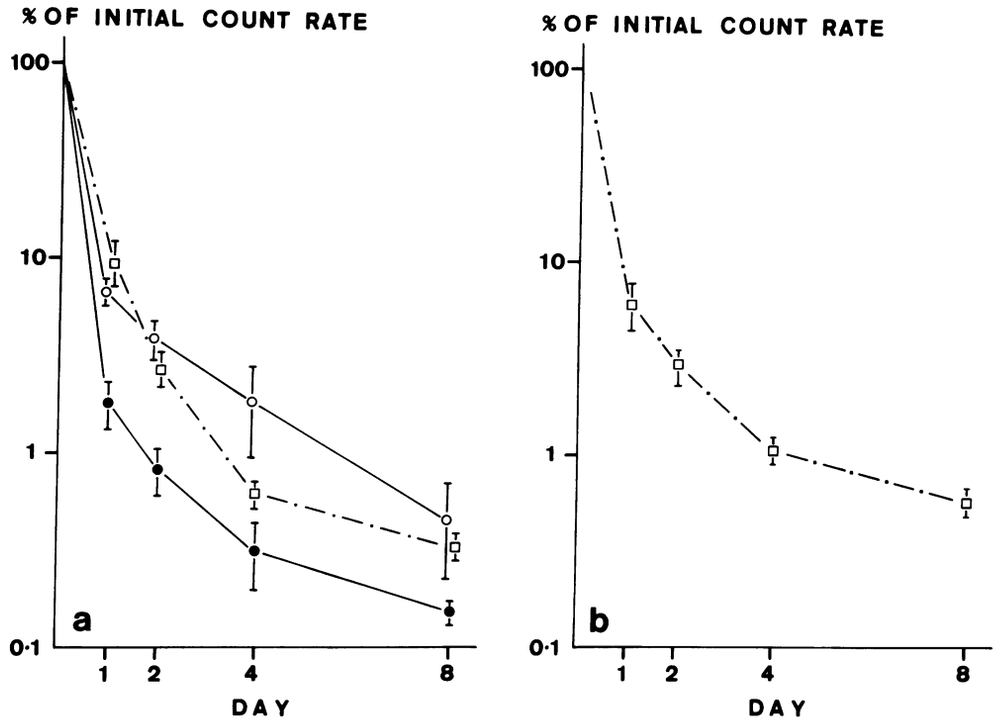


Figure 6. External radioactivity measurements at various days after intra-articular injection of 25 μg of ^{125}I-labeled proteins. Values represent the mean±SEM of groups of four rats. (○) Histone f1, (●) lysozyme, (□) Y.e. 0:3 19 kD. (*a*) Nonimmune rats. (*b*) Immune rats. Except at day 4, retention of Y.e. 0:3 19 kD is significantly higher compared to lysozyme (day 1, $P < 0.01$; day 2, $P < 0.02$; day 8, $P < 0.01$). Persistence of Y.e. 0:3 19 kD in immune animals is also significantly higher than in nonimmune rats at later time points (day 4, $P < 0.001$; day 8, $P < 0.01$). Retention values of histone f1 are not significantly different from those of Y.e. 0:3 19 kD at all timepoints.

The values obtained with Y.e. 0:3 19 kD were similar to those found with histone f1, which revealed the strongest affinity of those proteins tested to heparin-Sepharose. As shown here the binding behavior on this matrix can be used as an in vitro test for screening potential arthritogens on the basis of charge–charge interactions. The retention of lysozyme and Y.e. 0:3 19-kD protein in vivo in knee joints and in vitro on heparin-Sepharose reflect their markedly different arthritogenic potential: native lysozyme (a nonaggregating molecule) was shown to cause only a mild transient arthritis with a duration of less than 2 wk (Gondolf et al., submitted for publication), whereas the *Yersinia* antigen could induce chronic long-standing arthritis. Histone f1 could not be tested for its inflammatory potential in rats; because of the high level of conservation during evolution histones have limited immunogenic properties.

Antigen persistence of Y.e. 0:3 19 kD in immune rats was enhanced compared to the group of nonimmune animals; this suggests that immune complex formation was also a relevant factor for antigen retention and presumably for arthritogenicity, besides charge-based effects. In experiments using chemically cationized proteins in mice, van den Berg, van Lent, and co-workers (14–16) found that the retention of a polycation depended on the dose of antigen given, the type of substitution employed (methylation or amidation) and the immune status of the mice. Antigen retention is one of the decisive factors influencing the outcome of the inflammatory reaction induced, and is obviously a complex phenomenon that warrants further study.

The antigen under study is present in relative abundance intracellularly in Y.e. 0:3 (about 30 mg can be obtained from 50-g wet cells). It was also shown to occur in other Y.e. strains in much lower quantities, but was absent in a number of other enterobacteria also associated with reactive arthritis (*E. coli, S. enteritides, S. sonnei,* and *C. jejuni*). The protein under study has now been partially sequenced (not reported here), and a comparison with published sequences indicates that it has not been previously identified.

The function of the Y.e. 0:3 19-kD protein may be in binding (stabilization) of nucleic acids. Several observations support this conclusion: firstly, a major portion of the protein appears in the ribosomal pellet, when the microbes are disrupted and the membranes are removed by repeated centrifugation (17). Secondly, the protein has strong affinity for DNA-cellulose, which cannot be explained by a general nonspecific binding of DNA by cationic molecules, since lysozyme—also highly cationic (pI 11.3)—shows a markedly different elution behavior from DNA-cellulose matrix. In fact, the elution behavior of the Y.e. 0:3 19-kD protein was similar to that of histone f1, a well-studied DNA-packing protein in eukaryotic cells (31). Furthermore, the protein was also seen in the 150,000-g supernatant after excess heating and strong acidification (results not reported here), a fraction, where Lathe and co-workers (17) observed two DNA-binding proteins in high concentrations in a comparable preparation from an *E. coli* strain. Whether the Y.e. 0:3 19-kD protein belongs to the group of so called "histone-like proteins" (32, 33) or not, cannot be answered, since possession of specific characteristics of such proteins, such co-extraction of protein and DNA from low salt nucleoids (34) or more important the packing of DNA (supercoiling) (35–37) remains to be demonstrated. The extraction from the ribosomal pellet in our preparation is not contradictory to the proposed "histone-like" character, since the best-studied histone-like protein from prokaryotic cells—protein HU from *E. coli*—actually revealed a higher affinity for RNA than for DNA (38).

It is important to note that Y.e. 0:3 19 kD was not the only cationic intracellular molecule found in abundance in this species. It was chosen for experimental study because of its excellent immunogenicity in animals compared to other, equally cationic proteins; these other proteins may be more highly con-

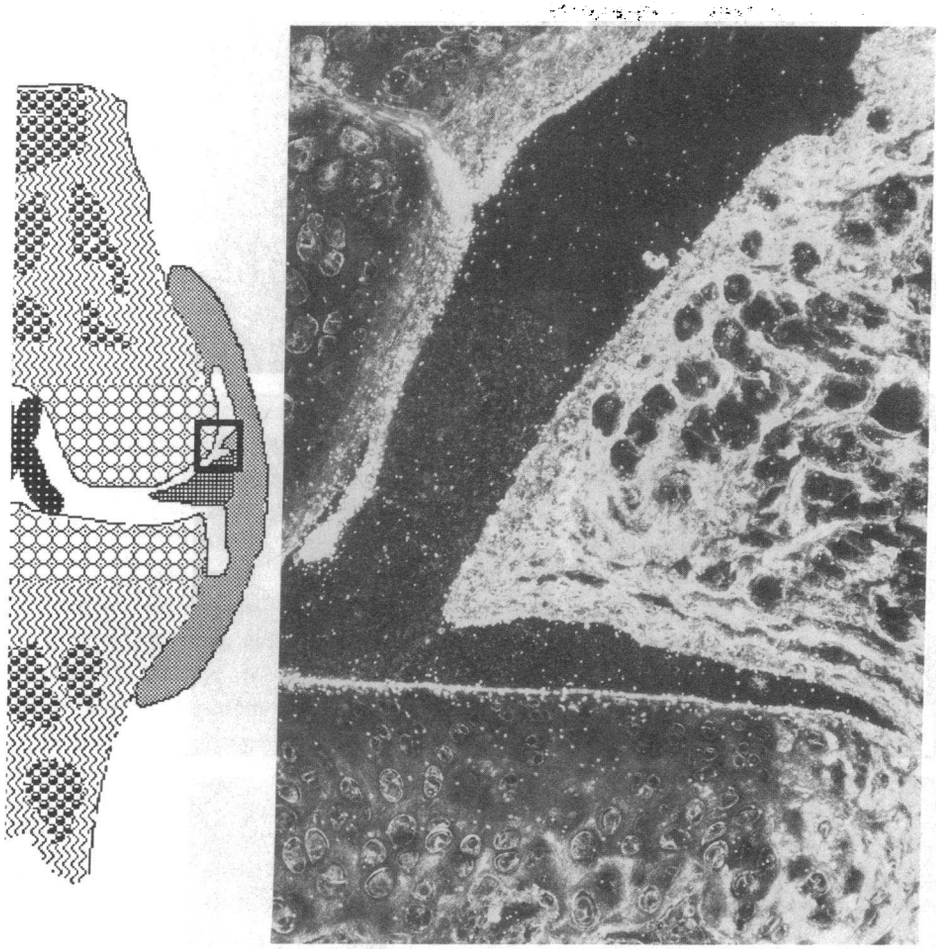


Figure 7. Autoradiograph of knee joint section of a rat 6 h after intra-articular injection of 250 μCi of ^{125}I-labeled Y.e. 0:3 19 kD. The schematic illustration (*left*) explains the anatomical situation. The antigen (*white spots*) is well retained in the synovium juxtaposed to the cartilage as well as in the cartilage and the meniscus (×50, H&E stain, dark-field).

served, like histones, and they initiate little if any immune response, even after coupling to a carrier such as keyhole limpet hemocyanin (unpublished observations).

The Y.e. 0:3 19-kD fraction employed to induce arthritis was in aggregated form (about 70 kD). The dimeric form (38 kD) alone produced little inflammation and this is further evidence that molecular size is of vital importance in the induction of tissue injury by a cationic protein. Previous studies have shown that binding and persistence of cationic antigens to joint structures increase dramatically when the size of the antigen exceeds 40–50 kD (15, and Gondolf et al., submitted for publication). The *Yersinia* protein was complexed with other protein(s) that were not identified, these are unlikely to have been involved in the induction of disease as they revealed little immunogenicity.

Currently the concept that reactive arthritis could be provoked by exogenous antigens in joints is attracting increased interest. Major progress in unravelling the pathogenesis of *Yersinia*-triggered arthritis was achieved by investigations performed in Finland (2–4, 39), where *Yersinia* infections and

Table I. Experimental Groups: Induction of Arthritis

Group (n = 6)	Immunization	Delayed-type hypersensitivity test‡	Antibody titer§	Intra-articular challenge‖
1	Y.e. 0:3 19 kD (65 kD-fraction I*b*)	47±16	10.2±1.4	50 μg of Y.e. 0:3 19 kD (65 kD)
2	Y.e. 0:3 19 kD (65 kD)	49±13	10.4±0.8	50 μg of lysozyme tetramer* (pI 11.3, 58 kD)
3	BSA	25±7	2.1±1.2	50 μg Y.e. 0:3 19 kD (65 kD)
4*	α-Lactalbumin polymer	35±16	10.4±0.9	50 μg α-lactalbumin polymer (pI 5.3, 58 kD)

* From Gondolf et al., manuscript submitted for publication. ‡ Mean values ± SD of increase of ear thickness (mm × 10^{-2}) measured at 48 h. § In ELISA, given as $\log_2$-values above means±3 SD of a normal collective (n = 6). ‖ In 50 μl of PBS into the left hindleg knee joint.

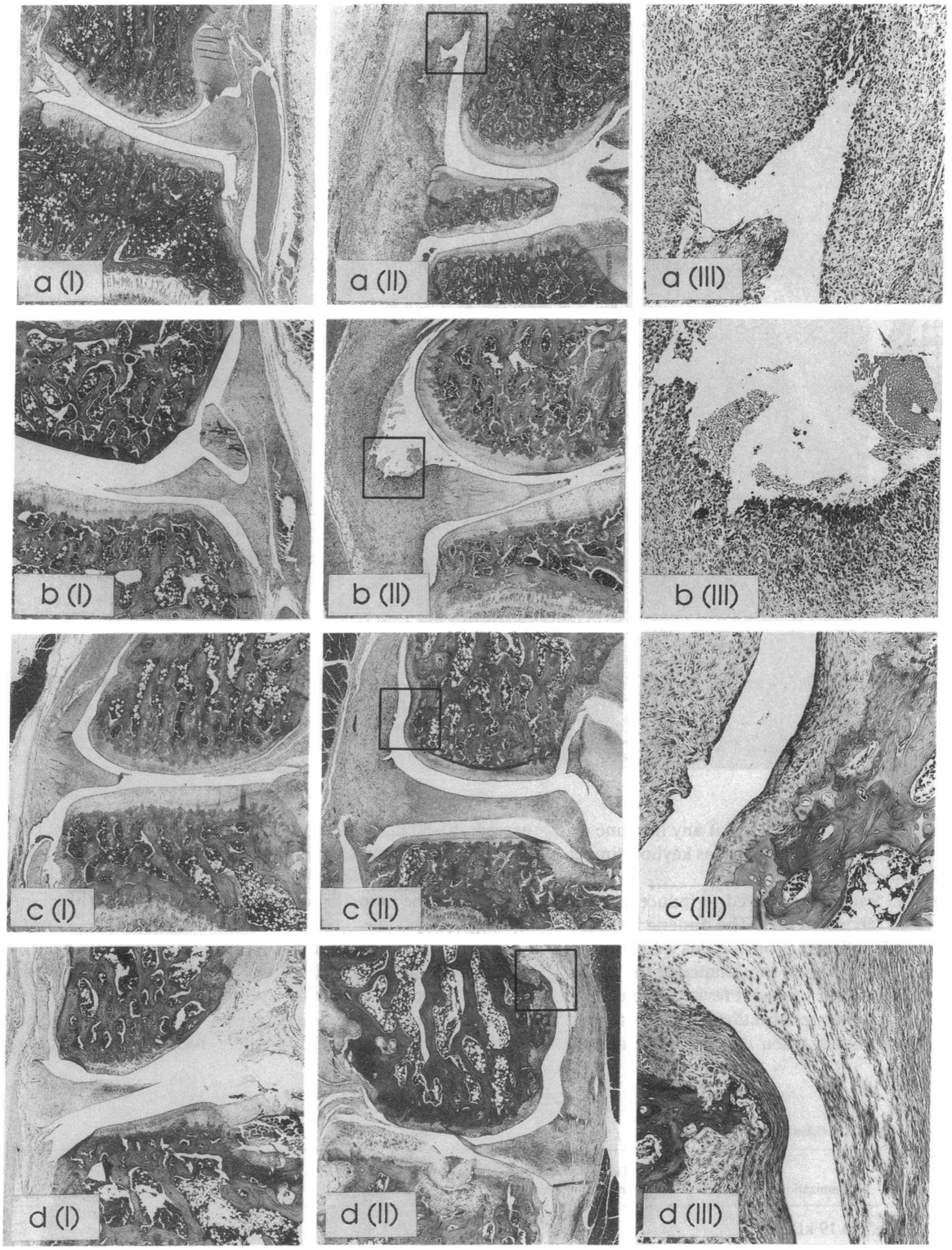


Figure 8. Histologic changes in the course of arthritis. Control (normal) right knee joint sections (I), and left (arthritic) knee joint sections (II and III [*inset*]) from previously immunized rats after intra-articular challenge of 50 μg of Y.e. 0:3 19 kD. (*a*) II, III, day 2: severe edema and neutrophilic granulocyte infiltrate are clearly seen within the synovium. (*b*) II, III, day 6: subacute inflammatory infiltrate of synovium and meniscus with developing pannus and beginning of destruction of the cartilage structures. (*c*) II, III, day 26: sclerosis with thickening of the synovium, paucity of inflammatory infiltrate and increase of fibroblasts indicate the chronic stage. (*d*) II, III, day 78: similar changes as at day 26 (*c*), in addition cartilage structures are partially replaced by fibrotic scar tissue. (Original magnification: I and II, ×13; III, ×70).

related diseases are widespread and receive close attention. These authors focused attention on bacterial components present within joint structures and were able to demonstrate antigens in synovial fluid cells (3), in circulating immune complexes (39) in immune complexes derived from synovial fluid (2), and probably in the synovium itself (4). Very recently the

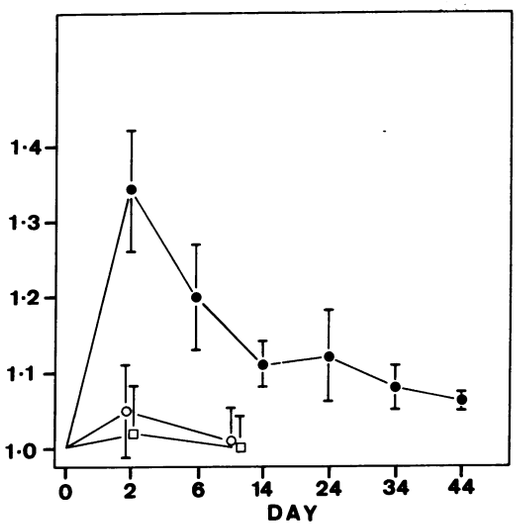


Figure 9. Left right ratio of ^{99m}Tc uptake at various days after intra-articular injection of 50 μg of Y.e. 0:3 19 kD into the left knee joint of previously immunized rats. (●) Chronic arthritis is seen (L/R ratios > 1.1). In contrast, the control animals—(○) BSA immunized rats, challenged with 50 μg of Y.e. 0:3 19 kD and (□) Y.e. 0:3 19 kD–immunized rats, challenged with 50 μg tetrameric lysozyme—show little inflammation. Data represent the mean±SD of six rats.

presence of the microbe itself in joints was reported in *Yersinia*-triggered arthritis (40). It is worth mentioning that immune complex formation in reactive arthritis in humans is a frequent feature, not only in the circulation (39) but more important within the joint (2).

In the early phase of Reiter's syndrome interesting findings were reported by several investigators: Schumacher et al. (5) were able to show, by immune electron microscopy, strongly positive peroxidase reaction products in vacuoles of perivascular cells, which were highly suggestive of chlamydial elementary bodies. These authors also found fibrin and electron-dense granular material in the vessel walls, which could have been immune complexes. Additionally, Baldassare and co-workers (7) noted deposition of IgM, fibrin, and C3 around the vessels in the synovium by immunofluorescence in the majority of 12 patients with Reiter's syndrome. The latter findings are a focus of special interest, inasmuch as they indicate a possible way for initiation of the disease: the deposition of immunoglobulins around the vessels in the synovium could occur as an immune response to a previously bound antigen, which, for instance, could have been released from bacteria within the circulation. This view is supported by an experimental observation with the Y.e. 0:3 19-kD protein: on perfusion into the renal artery of rats, the protein showed strong affinity not only for the glomerular basement membrane, but also for the blood vessels in general (e.g., peritubular vessels) in a fine granular pattern by immunofluorescence; those deposits were accessible for circulating antibody (manuscript in preparation). This justifies the speculation that cationic *Yersinia* products (e.g., histone-like proteins), once released into the circulation, could accumulate within the vessel walls of the synovium, acting thereby as a target for immune complex formation and immune reactions in general.

The vast majority of individuals with proven serotype 0:3 infections recognized the Y.e. 0:3 19-kD protein in either western blot or in ELISA or both, whereas sera of healthy individuals usually did not react with the antigen. This result shows that the antigenic fraction studied here is expressed during *Yersinia* infection in man and is immunogenic. The question of involvement of the Y.e. 0:3 19-kD antigen in cases of *Yersinia*-induced reactive arthritis in patients is now under study.

Serologic studies may well provide useful information as Granfors, Toivanen, and co-workers (41–43) found a more vigorous and prolonged serum-IgA and IgG anti–Yersinia antibody response in arthritic compared to nonarthritic patients. Elevated IgA levels indicate a continuous stimulation of the immune system by non- or ineffectively eliminated antigens or whole microbes; moreover, it has been speculated that the inability of IgA antibodies to directly fix complement (44, 45), in contrast to IgM and IgG, and their specific binding to antigenic sites might result in a blocking of phagocyte function and impair antigen elimination (46).

Several ideas are currently being connected with the pathogenesis of reactive arthritis. A new concept of stress protein induced autoimmunity and rheumatoid arthritis (47) has claimed attention, since stress proteins are highly conserved during evolution and occur in many bacterial species (48). This notion could also be helpful in explaining the induction of reactive arthritis by a variety of bacterial species. Another important observation in cases of reactive arthritis was the detection of a genetic component at the host level. The HLA-B27 antigen occurs in ~ 75% of patients (1, 49), compared to < 10% in the normal population. Various hypotheses have been proposed to explain this connection, including the concept of molecular mimicry (50, 51) which concerns structural similarities between bacterial components and HLA-B27 antigen. To date partial sequence data did not reveal any homologies between HLA-B27 and Y.e. 0:3 19 kD.

Many questions remain open, but after paying due homage to the convincing immunological and histopathological findings in reactive arthritis cited above, we favor the "classic" concept of deposition of bacterial components or survival of bacteria itself within structures. The current work delineates a role for naturally occurring cationic antigens in the induction of arthritis. We speculate further that nucleic acid binding proteins, especially histone-like proteins from prokaryotic cells could be involved in the pathogenesis of reactive arthritis.

Acknowledgments

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