# *Electrical Charge and Joint Inflammation*

## *Suppression of Cationic aBSA-Induced Arthritis With a Competitive Polycation*

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**Chronicity of murine allergic arthritis depends on the charge-mediated retention of the cationic antigen in the joint. The authors examined whether arthritis induced with the positively charged antigen amidated bovine serum albumin (aBSA) could be modulated with a nonimmunogenic polycation by competition for anionic retention sites in the joint. Concomitant intraarticular injection of aBSA with the cationic protein protamine chloride (pI~10) strongly reduced the retention of aBSA. Detailed analysis revealed that the retention of aBSA in the noncartilaginous tissues was significantly reduced by protamine, whereas the retention in the highly negatively charged cartilage was completely prevented. Joint inflammation was already significantly suppressed at Day 3 and suppression was still demonstrable at Day 28. Protamine treatment also caused a highly significant reduction in cartilage damage and bone apposition. Control experiments indicated that the suppressive effect of protamine was related to its interference with antigen retention in the joint and not to a mere antiinflammatory action. (Am J Pathol 1987, 127:15–26)**

ANTIGEN-INDUCED arthritis, which serves as a model for chronic immunologic joint inflammation, is elicited by intraarticular injection of antigen into the knee joint of preimmunized animals. The chronicity of antigen-induced arthritis has been described to depend on sufficient antigen retention in the joint, together with adequate delayed type hypersensitivity against the retained antigen.[1,2] Mechanisms operative in antigen retention in the joint are *in situ* immune complex formation within the collagenous tissues of the joint[2,3] and, as recently found in our laboratory, charge-mediated binding.[4–6] Cationic antigens like methylated or amidated bovine serum albumin (mBSA and aBSA, respectively) show a high affinity for the negatively charged joint structures, in particular the articular cartilage, and with these antigens a chronic arthritis can be induced in immunized mice.[4]

Apart from its potential role in joint inflammation, electrical charge has recently emerged as an important new concept in renal immunopathology. Membranous nephropathy may develop in immunized animals upon systemic administration of cationic antigen, and this appeared to be due to deposition of the positively charged antigen at fixed anionic sites of the glomerular capillary wall, followed by binding of immunoglobulins and complement.[7,8] The importance of charge was further substantiated by the prevention of *in situ* immune complex formation with protamine,[9] a substance with weak immunogenicity, highly cationic charge (pI~10), and low molecular weight (7000). Recent *in vitro* work from our laboratory indicated that competitive cationic proteins like mBSA or protamine can almost completely prevent the retention of cationic aBSA in hyaline articular cartilage. Their effects on aBSA retention in noncartilaginous joint tissues were less pronounced.[6]

The present study was focused first to clarify the general validity of the importance of electrical charge in murine joint inflammation. It was shown that the induction of chronic allergic arthritis with cationic

Accepted for publication October 22, 1986.

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antigen not only holds for cationic BSA but also for cationic aOA (amidated ovalbumin), and that aBSA-induced arthritis can be elicited in various strains of mice. Second, we examined whether a competitive cationic protein could modulate the arthritis by competition for anionic retention sites in the joint. In general, suppression of joint inflammation is approached by therapy with antiinflammatory, immunosuppressive, or second-line drugs like gold or D-penicillamine, and it seemed worthwhile to extend this approach to modulation of the antigenic load, using the competitive polycation protamine. Control experiments were performed to substantiate that protamine exerts its effect by interference with antigen retention and not by a mere antiinflammatory action.

## Materials and Methods

### Animals

Unless stated otherwise, experiments were performed with male C57Bl mice, aged 6–8 weeks at the start of the immunization protocol, or aged 10–15 weeks when used nonimmunized. The animals were bred in our own animal house (Overasselt, The Netherlands). In one experiment three strains were compared: C57Bl mice (H-$2^b$); CBA mice (H-$2^q$) and BALB/c mice (H-$2^d$), the latter strains obtained from TNO, Rijswijk, The Netherlands. Of notice, these CBA mice are of the *q* haplotype instead of the usual H-$2^k$. Animals injected with $^{125}$I-antigen were given water containing KI (50 mg/l) 2 days before injection and maintained on this water thereafter.

### Antigens

OA, BSA, and mBSA were obtained from Sigma Chemical Co., St. Louis, Missouri. OA and BSA were used unmodified as native proteins or used as a substrate for the preparation of amidated proteins as described,[4,10,11] with 1-ethyl-3 (3 dimethyl-aminopropyl) carbodiimide hydrochloride (EDC, Sigma) as activator, and N,N-dimethyl-1,3-propane diamine (DMPA, BDH Chemicals, Ltd., Poole, England) as a nucleophile. The isoelectric point (pI) of the proteins was determined with isoelectric focusing: pI of aBSA, approximately 8.5–9; pI of oA, approximately 9.

### Iodination of Antigens

$^{125}$I labeling was performed by the chloramine T method.[12] $^{125}$I-antigen was separated from free $^{125}$I by Sephadex G25 fractionation. The specific activity was 2–3 $\mu$Ci/$\mu$g.

### Immunization

Mice were immunized with 100 $\mu$g antigen in 0.1 ml Freund's complete adjuvant (FCA) emulsion (Difco Laboratories, Detroit, Mich) on Days 0 and 7 by divided injections into the flank skin and the footpads of the fore legs. On both occasions, $2.10^9$ heat-killed *Bordetella pertussis* organisms (National Institute of Public Health, Bilthoven, The Netherlands) were administered intraperitoneally as an additional adjuvant.

### Skin Testing

Delayed hypersensitivity was measured at 24 and 48 hours after injection of 5 $\mu$g antigen in 10 $\mu$l saline into the pinna of the ear. Increase in ear thickness was measured with an engineer's micrometer. aBSA and mBSA induced a small nonspecific reaction in nonimmune mice at 24 hours ($5 \times 10^{-2}$mm). Values in immune mice are corrected for this background. Strong delayed hypersensitivity is measurable for both aBSA and mBSA at 2–3 weeks after immunization, and delayed hypersensitivity remained high till at least 3 months after immunization (unpublished observations).

### Antibody Determination

Antibodies were measured with an ELISA assay[4] with the use of 50% of the maximal extinction as end point.

### Arthritis Induction

Sixty micrograms of antigen (6 $\mu$l of a 10 mg/ml protein solution) was injected into the right knee joint of mice immunized 3 weeks before. The left knee joint was given an injection of 6 $\mu$l saline as a control. Inflammatory reactions or joint damage was never seen in these left knee joints.

### Protamine Experiments

To determine the optimal dose of the competitive polycation protamine chloride (7000 mol wt, pI~10; purchased from Sigma Chemical Co.) to modulate the retention of aBSA in the knee joint, we used various protamine–aBSA dosage combinations. The first experiments were done in nonimmune mice: 6 $\mu$l of a protamine solution (1–100 mg/ml) was injected into the right knee joint, and OA was injected into the left joint with a protein concentration similar to that in the contralateral protamine-treated joint. Two hours later we injected 6 $\mu$l $^{125}$I-aBSA (1–10 mg/ml) into both joints. Retention of aBSA was measured at

various days thereafter by external gamma counting over the knee joints (see below). The retention in the protamine-treated joint was expressed as percentage of the retention in the contralateral OA-treated joint. The highest dose of protamine (100 mg/ml) caused some inflammation, as measured with $^{99m}$Tc uptake (R/L ratio, 1:2 at Day 2) and confirmed with histologic studies. Cartilage and bone damage was not observed. This high protamine dose was not further used. The combination 30 mg/ml protamine and 10 mg/ml aBSA was chosen for study of the protamine effect in immunized mice. From a group of 30 immunized mice 10 were used for the retention experiments as described above. One joint was treated with OA/$^{125}$I-aBSA and the contralateral joint with protamine/$^{125}$I-aBSA. The other 20 mice received injections on the same day of OA/aBSA (10 mice) or OA/protamine (10 mice) in the right knee joint, whereas the left joint was treated with saline. This induction of unilateral arthritis enables proper quantitation of the severity of the joint inflammation with the $^{99m}$Tc uptake method (see below). The animals were sacrificed for histologic study of the joint at Day 21. In a second experiment 15 mice were given aBSA with either OA or protamine, and groups of animals were sacrificed at Days 14 and 28.

### Antigen Retention Measurements

$^{125}$I-labeled antigen (6 $\mu$l) was injected into both knee joints. $^{125}$I radioactivity of the knee joints was measured by external gamma counting as described for $^{99m}$Tc uptake measurements (see below), and antigen retention was expressed as the percentage of the initial count rate measured immediately after intraarticular injection.

To discriminate between the effect of protamine on the retention of aBSA in cartilage and other joint tissue, we removed whole patellae with a standard amount of surrounding tissue from the joint at various days after $^{125}$I-aBSA injection. The specimens were fixed in 10% formalin and decalcified in 5% formic acid to enable proper dissection of the patella from the surrounding tissue. This assay has been developed by us for study of antigen and immune complex retention in cartilage and collagenous tissues.[4,6] The amount of antigen retained in the tissue is expressed per milligram wet tissue. Antigen retention in the cartilage is expressed per cartilage of the whole patella, which is a defined anatomical entity. Earlier autoradiographic studies made it clear that the retention of aBSA in the patellar cartilage is representative for retention of aBSA in other hyaline articular cartilage structures of the joint.[5]

### $^{99m}$Tc Uptake Measurements

Joint inflammation was determined by $^{99m}$Tc pertechnetate uptake measurements of the knee joints. The method has been described in detail elsewhere.[13] In brief, 10 $\mu$Ci $^{99m}$Tc was injected subcutaneously in the neck region, and 30 minutes later the gamma radiation over the knee joints was measured with a thallium-activated NaI scintillation crystal, with careful shielding of the radioactivity of the rest of the body. The animals were sedated 10 minutes before the measurement by intraperitoneal administration of 0.1 ml 4.5% chloralhydrate/10 g body weight. Arthritis was scored as the ratio of the $^{99m}$Tc uptake in the right versus that in the left knee joint (R/L ratio). R/L ratios exceeding 1.1 were taken to indicate inflammation of the right knee.[13]

### Histology

Knee joints were removed *in toto* and fixed in 10% phosphate-buffered formalin. After decalcification of the joints in 5% formic acid for 3 days, the tissues were processed and embedded in paraffin wax. Total knee sections (6 $\mu$) were prepared with the use of a frontal section plane.[5,14] At least 5 sections were prepared from a standardized area of the joint, which included the menisci, cruciate and collateral ligaments, and the patella. Joint inflammation was scored with the use of the parameters infiltrate in the joint tissue and exudate in the joint cavity. Both parameters were scored on a scale of 0–3, according to the amount of inflammatory cells present. Tissue destruction was scored with the use of the parameters chondrocyte death and bone apposition, as described previously[15,16]: 0, no chondrocyte death; 1, loss of one quarter of the chondrocytes; 2, loss of one half of the chondrocytes; 3, loss of all chondrocytes. Bone apposition was scored 0–3, according to the extent of new bone formation at lateral sites of the femur and the patella. The sections were scored by two investigators without knowledge of the experimental groups to which the specimens belonged.

### Autoradiography[5,14]

Histologic joint sections were mounted on gelatin-coated slides, dipped in K5 emulsion (Ilford, Basildon, Essex, England), and exposed for 2–8 weeks. After this period the slides were developed and stained with hematoxylin and eosin.

### Zymosan Arthritis

Zymosan can induce joint inflammation in non-immune animals.[17,18] To investigate the effect of protamine on zymosan-induced arthritis, we gave mice

Table 1 — Arthritis Induced With Cationic Ovalbumin (aOA)

| Antigen | pI | Immunization | R/L ratio of $^{99m}$TC uptake* | | | |
|---|---|---|---|---|---|---|
| | | | Day 2† | Day 4 | Day 7 | Day 14 |
| OA | 4.5 | — | 1.14 ± 0.07‡ | ND | 1.01 ± 0.04 | 0.96 ± 0.18 |
| OA | 4.5 | OA/FCA | 1.18 ± 0.14 | ND | 1.08 ± 0.04 | 1.04 ± 0.06 |
| aOA | 9.0 | — | 1.08 ± 0.11 | 0.99 ± 0.04 | 1.01 ± 0.08 | 0.95 ± 0.06 |
| aOA | 9.0 | aOA/FCA | 1.61 ± 0.18 | 1.82 ± 0.17 | 1.46 ± 0.20 | 1.21 ± 0.10 |

*Inflammation is expressed as the ratio of the Tc uptake in the right inflamed joint versus that in the left control joint. R/L ratios > 1.1 were taken to indicate joint inflammation.[13]

†Days after intraarticular injection of 60 μg of the antigen into the right knee joint.

‡Values represent the mean ± SD of groups of at least 5 mice.

intraarticular injections of 6 μl zymosan (30 mg/ml) together with protamine, OA, or aBSA (30 mg/ml). Zymosan was obtained from Koch-Light, Colnbrook, Bucks, England.

### Effect of Protamine on Cartilage Chondrocyte Function

Protamine (30 mg/ml) was injected intraarticularly into the right knee joint of nonimmune mice, whereas the left joints were treated with OA (30 mg/ml) as control protein. Patellae were isolated from groups of 5 mice at Days 3, 7, and 14 and incubated *in vitro* with $^{35}$S-sulfate (10 μCi/ml) for 2 hours. The $^{35}$S-proteoglycan synthesis was quantified by scintillation counting.[15,19]

## Results

### Induction of Arthritis With Cationic Ovalbumin

The severity of joint inflammation induced with either native ovalbumin (OA) or cationic amidated OA (aOA) in immune and nonimmune mice is shown in Table 1. Long-lasting joint inflammation is only induced with aOA in immunized mice. In agreement with our observations on BSA and aBSA, its cationic derivative,[4] cationic aOA was also much better retained in the joint, compared with native OA. Using $^{125}$I antigens, external gamma counting revealed that a more than 10 times higher amount was retained at Day 4 after injection (data not shown).

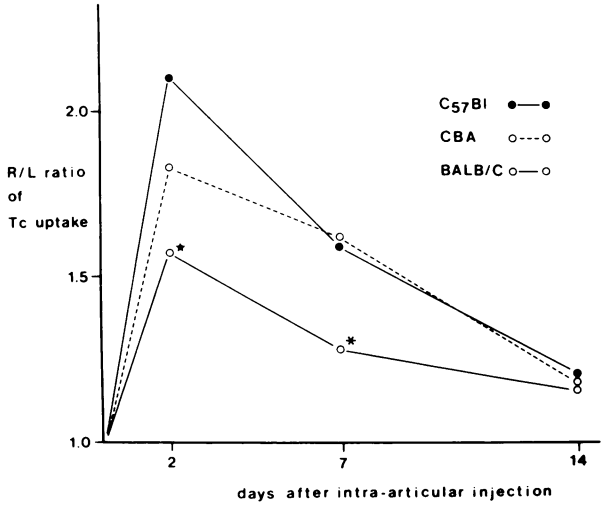



**Figure 1**—R/L ratios of $^{99m}$Tc uptake at various days after arthritis induction with 60 μg aBSA in immunized mice. Significant differences (Mann-Whitney): at Day 2 $P < 0.05$ BALB/c versus CBA, $P < 0.01$ BALB/c versus C57Bl; at Day 7 $P < 0.01$ BALB/c versus CBA or C57Bl.

### aBSA Arthritis in Various Strains

Figure 1 shows the course of the arthritis induced with cationic aBSA in immunized C57Bl, CBA, and BALB/c mice. A clear, long-lasting arthritis was found in all 3 strains, although the severity of the joint inflammation was definitely less in BALB/c mice at Days 2 and 7. Table 2 shows the antigen retention in the joint. The initial clearance of $^{125}$I-aBSA was similar for all 3 strains, but from Day 7 on, higher amounts were retained in the joints of BALB/c mice. The latter may be compatible with the less severe joint inflammation in BALB/c mice, because we have recently shown that joint inflammation augments antigen clearance.[4] The histologic features of the various phases of the arthritis are shown in Figure 2. A massive infiltration of the synovial tissue and exudation in the joint cavity was found in the early stages, followed later on by bone erosion as well as bone apposition and cartilage destruction. The arthritis at Day 21 was scored semiquantitatively with the use of a num-

Table 2 — Antigen Retention in the Knee Joints of Immunized Mice of Various Strains

| Strain | Antigen retention (% of the initial dose)* | | | | |
|---|---|---|---|---|---|
| | Day 2† | Day 4 | Day 7 | Day 14 | Day 28 |
| C57B1 | 7.8‡ | 2.9 | 1.2 | 0.45 | 0.39 |
| CBA | 9.4 | 2.6 | 1.3 | 0.45 | 0.35 |
| BALB/c | 8.2 | 3.0 | 1.9§ | 0.72§ | 0.65§ |

*Measured by external gamma counting.

†Days after intraarticular injection of $^{125}$I-aBSA (60 μg).

‡Values are the mean of groups of 5 mice.

§Significant difference between values in BALB/c mice and the two other strains ($P < 0.01$, Mann-Whitney).

ber of histologic parameters (Table 3). Ongoing arthritis was manifest in all three strains, with perhaps the most active character in BALB/c mice, as demonstrated by the high score for exudate and the relatively large numbers of granulocytes present in the synovium of BALB/c mice at this late stage of the arthritis (Figure 2F). Cartilage damage and new bone formation was higher in C57B1 and CBA mice, probably reflecting the more severe preceding inflammation in these two strains.

Because the severity and chronicity of antigen-induced arthritis may be related to antigen retention, and the presence of cell-mediated immunity against the retained antigen, the immune status was determined. All strains of mice develop significant delayed type hypersensitivity reactions, and the antibody titers against aBSA were similar (Table 4).

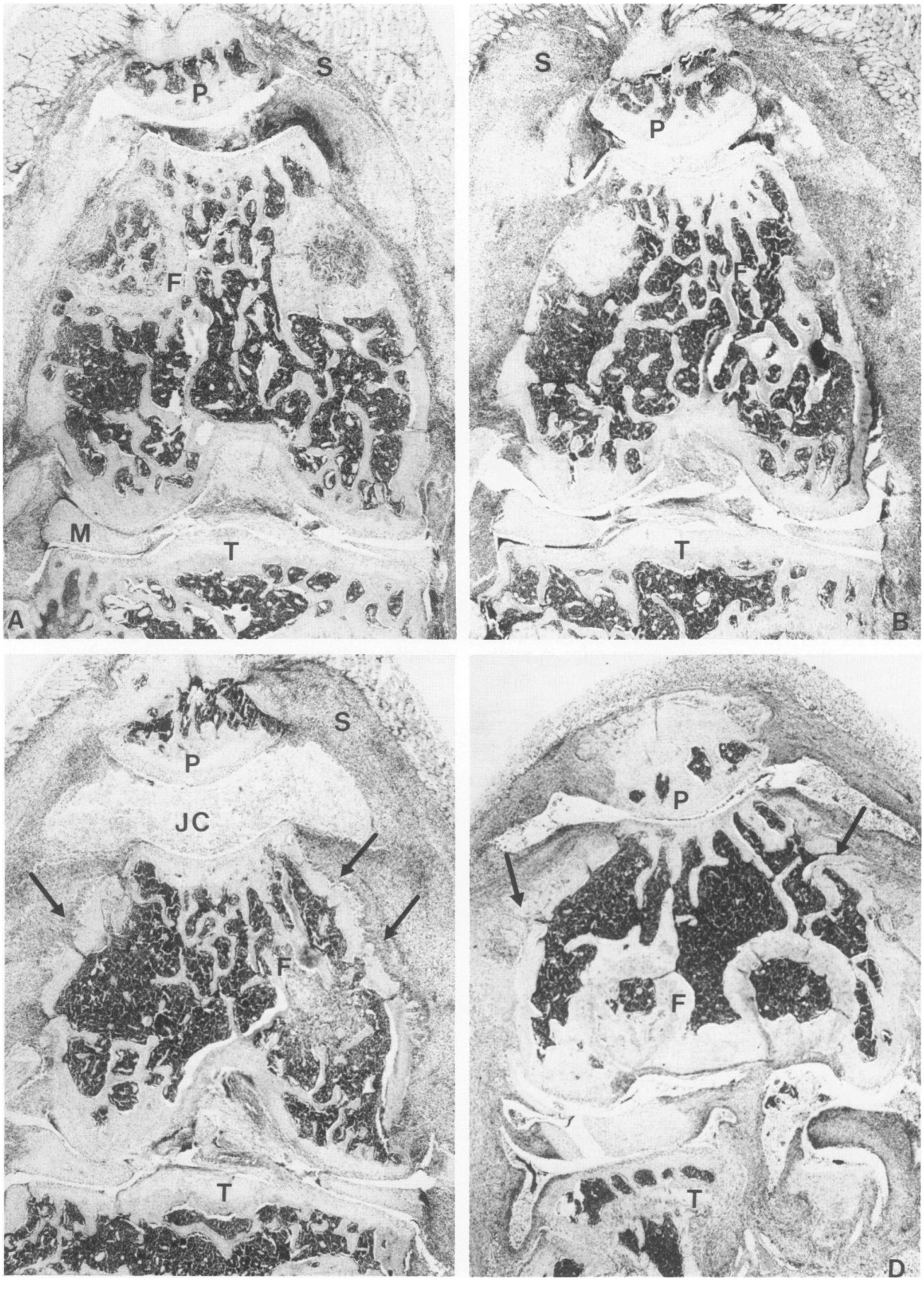
### Effect of a Competitive Polycation on Antigen Retention

We have previously shown that protamine, a low-molecular-weight polycation (7000 mol wt; pI~10), can prevent retention of aBSA in articular cartilage *in vitro*.[6] To investigate whether protamine can be used to modulate arthritis by interference with antigen retention *in vivo*, we first studied the effect on $^{125}$I-aBSA retention in the joints of nonimmune mice. A number of protamine–aBSA dosage combinations were used. Intraarticular injection of 6 $\mu$l of a protamine solution of 10 mg/ml 2 hours before the injection of $^{125}$I-aBSA (1 mg/ml) could not prevent the retention of the latter (Table 5). However, a protamine dose three times higher (30 mg/ml) clearly suppressed the retention of aBSA, and this effect was even more pronounced when protamine and $^{125}$I-aBSA were injected simultaneously. A protamine concentration of 100 mg/ml also caused strong suppression (Table 5), but this high dose seems unsuitable, because alone it induced significant joint inflammation in nonimmune mice. The dose of 30 mg/ml was therefore chosen for investigation of its effect on the retention of aBSA in immune mice. One group of mice was given $^{125}$I-aBSA and protamine, whereas a second group received $^{125}$I-aBSA, together with OA as a noncationic control protein. Table 6 shows that protamine also suppressed the retention of aBSA in immune mice. The differences, however, were less pronounced, probably related to the fact that antigen retention is already decreased in immune, compared with nonimmune, mice because of the development of joint inflammation.[4]

To discriminate between the effect of protamine on the retention of aBSA in cartilage and joint tissue, whole patellae with a standard amount of surrounding tissue were removed from the joints at various days after antigen injection. Protamine almost completely prevented the retention of aBSA in the patellar cartilage of nonimmune mice (2%), whereas the retention in the tissue was reduced to about 10% (Table 7). Protamine also completely prevented the retention of aBSA in the patellar cartilage of immune mice. The percentual suppression, compared with that in OA-treated mice, was, however, less in immune versus nonimmune mice. Because of the development of joint inflammation in the immune mice, the patellar cartilage becomes depleted of proteoglycans, which results in loss of anionic binding sites[4,6] and therefore in antigen retention. This tendency became clearer at later days. On the tissue level an effect of protamine on aBSA retention in immune mice was obvious at Day 2 (33%) but had completely disappeared at Day 7. This is caused by the vigorous clearance of aBSA from this compartment by the inflammatory process: 400 cpm in the nonimmune OA group at Day 7 versus 57 cpm in the immune OA group (Table 7). This clearance phenomenon was also demonstrated in a semiquantitative way in our previous autoradiographic study.[5] The complete prevention of $^{125}$I-aBSA retention by protamine on the cartilage level was further substantiated by autoradiography on whole joint sections (Figure 3).

### Effect of Protamine on aBSA-Induced Arthritis

In parallel with the retention experiments with $^{125}$I-aBSA similar groups were given intraarticular injections of nonlabeled aBSA together with either protamine or OA. Joint inflammation, as measured by $^{99m}$Tc uptake, was significantly suppressed in the protamine-treated group (Figure 4). This effect was clear-cut at Day 3 and remained present till the end of the period studied. Histologic studies of whole joint sections taken at Day 21 confirmed the suppression of joint inflammation and further revealed a highly significant decrease in cartilage damage and bone apposition (Table 8). In a second experiment similar suppression of $^{99m}$Tc uptake was found (data not shown), and histologic studies were done on Days 14 and 28. Although low numbers were compared at Day 14 ($n = 5$), highly significant suppression was found for all histologic parameters (Table 8). At Day 28 sustained inflammation was evident in the OA group and suppression in the protamine-treated group was still demonstrable. A representative histologic picture of arthritis in the control group and the protamine-treated group is shown in Figure 5.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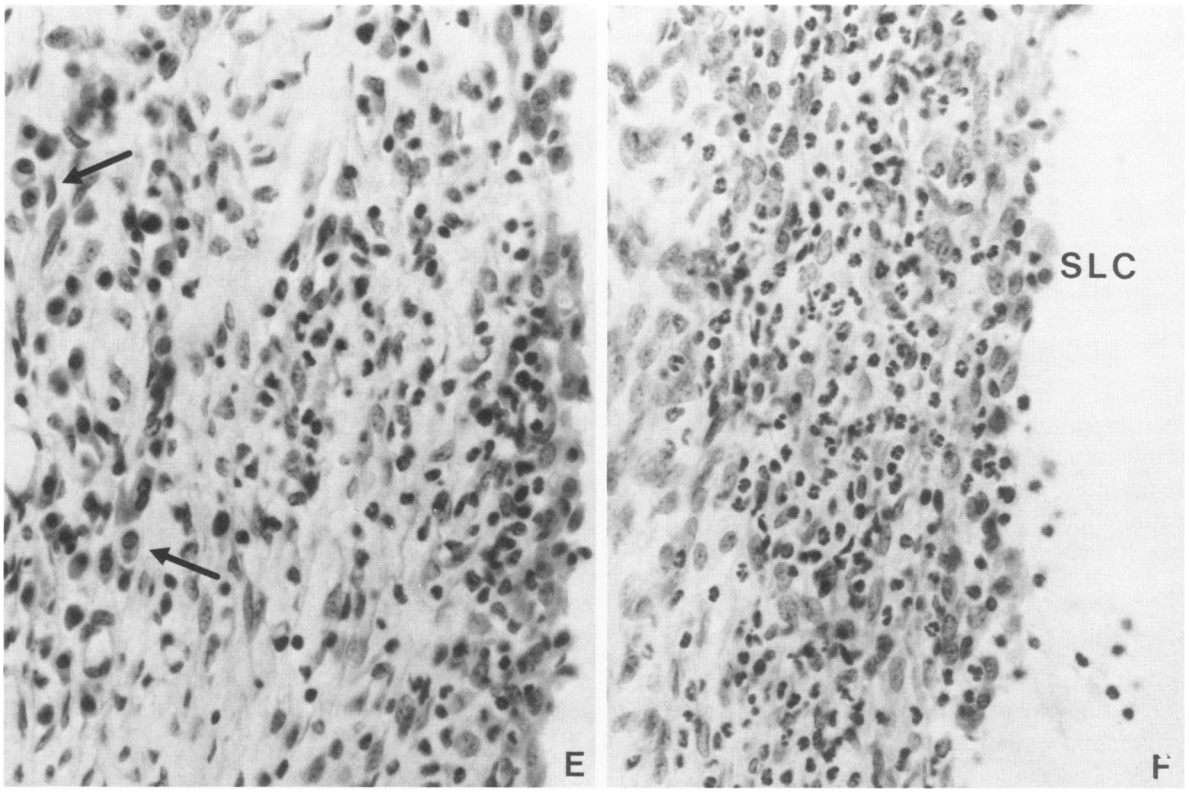


**Figure 2 A–D**—Whole joint sections (×30) of aBSA-induced arthritis in C57B1 mice at Days 2 (**A**), 4 (**B**), 7 (**C**), and 14 (**D**). **E–F**—Details of the synovium (×200) of a C57Bl (**E**) and BALB/c mouse (**F**) at Day 21. At Day 2 most inflammatory cells are found in the exudate in the joint cavity; later, infiltration in the synovial tissue is the most salient feature. Note the irregular lining of the periosteum at Day 7 (*arrows*) due to activation of osteogenic cells, resulting in pronounced new bone formation at Day 14 (*arrows*). At Day 21 (**E**) numerous plasma cells are present, predominantly found in the deeper layers of the synovial tissue (*arrows*). **F**—Granulocyte-rich infiltrate in the synovial tissue of a BALB/c mouse at Day 21. *P*, patella, *F*, femur; *T*, tibia, *M*, meniscus; *S*, synovium; *JC*, joint cavity; *SLC*, synovial lining cells.

## Control Experiments

To obtain evidence that the suppressive effect of protamine was related to an effect on antigen retention and not to a mere antiinflammatory effect, we investigated its effect on zymosan-induced arthritis.[17] Concomitant intraarticular injection of zymosan with protamine did not suppress the inflammation (Table 9).

In addition, the effect of protamine was tested in a delayed type hypersensitivity reaction. We chose mBSA as the antigen because earlier studies had revealed that protamine does not interfere with the retention of mBSA,[6] and a potential effect of protamine could then be attributed solely to an antiinflammatory effect. Table 10 shows that protamine was also ineffective in this type of inflammation.

Because highly cationic proteins like protamine (pI~10) are potentially toxic, we furthermore tested its effect on chondrocyte synthetic function in articu-

Table 3—Histology of aBSA-Induced Arthritis (Day 21)

| | | Histologic scores * | | | |
|---|---|---|---|---|---|
| | | Inflammation | | Joint damage | |
| Strain | n | Infiltrate | Exudate | Chondrocyte death | Bone apposition |
| C57Bl | 9 | 1.75† | 0.83 | 2.20 | 2.15 |
| CBA | 8 | 1.38 | 0.47 | 1.88 ‖ | 1.38 § |
| BALB/c | 10 | 1.40 | 1.10‡ | 1.00 | 1.05 |

*Scored on a three-point scale (see Materials and Methods).
†Mean values of groups of 8–10 mice.
‡$P<0.05$ (Mann–Whitney).
§$P<0.025$ (Mann–Whitney).
‖$P<0.01$ (Mann–Whitney).

Table 4 — Delayed Hypersensitivity and Antibody Levels in Mice Immunized With aBSA/FCA

| Strain | Ear test (mm × $10^{-2}$)* 24 hours | Ear test (mm × $10^{-2}$)* 48 hours | Antibody titer† (2 log) |
|---|---|---|---|
| C57Bl | 38 ± 8‡ | 49 ± 11 | 10 ± 1 |
| CBA | 19 ± 4 | 23 ± 6 | 9 ± 1 |
| BALB/c | 25 ± 4 | 32 ± 5 | 10 ± 2 |

*Skin test in the ear with 5 μg aBSA at Day 21 after immunization; values represent the increase in ear thickness.

†Determined with an ELISA, using 50% of the maximal extinction as the end point.

‡Values are the mean + SD of groups of 5 mice.

Table 6 — Effect of Concomitant Injection of Protamine on the Retention of $^{125}$I-aBSA in Immunized Mice

| Day | Antigen retention (%)* Protamine | Antigen retention (%)* OA | Relative† retention (%) |
|---|---|---|---|
| 1 | 10.4 ± 2.3 | 23.1 ± 4.1 | 45 |
| 2 | 2.0 ± 0.6 | 7.7 ± 3.1 | 26 |
| 4 | 0.78 ± 0.25 | 1.87 ± 0.38 | 42 |
| 7 | 0.43 ± 0.16 | 0.52 ± 0.19 | 82 |
| 14 | 0.19 ± 0.08 | 0.29 ± 0.10 | 65 |

Joints received injections of either protamine or OA (30 mg/ml) and $^{125}$I-aBSA (10 mg/ml).

*Retention is expressed as the percentage of the initial count rate measured immediately after intraarticular antigen injection.

†Retention in protamine- compared with OA-treated joints.

lar cartilage of the patella. Mice were given intraarticular injections of protamine (30 mg/ml), and patellae were isolated at Days 3, 7, and 14. We found no harmful effect on the chondrocyte proteoglycan synthesis as measured by $^{35}$S-sulfate incorporation.[15,19]

## Discussion

In this study we have shown that allergic joint inflammation induced with a cationic antigen can be suppressed by a competitive polycation. It was demonstrated that the suppressive effect of the polycation protamine was caused by its interference with antigen retention in the joint, and not by a mere antiinflammatory action.

In previous studies evidence was presented that electrical charge plays an important role in murine chronic arthritis. Long-lasting arthritis could be induced in immunized mice by intraarticular injection of cationic antigens related to the excellent charge mediated retention of such antigens in the joint.[4,5]

Cationic proteins show a high affinity for the highly negatively charged cartilage structures, and, to a lesser extent, for all connective tissues.[5,6] In vitro studies with the competitive polycation protamine suggested that the retention of aBSA in the cartilage could easily be prevented, but that the retention in other joint structures might well be less sensitive for charge-mediated competition.[6] The present *in vivo* data further substantiate this view. Retention of aBSA in the patellar cartilage was completely prevented by concomitant intraarticular injection of protamine, both in immune and nonimmune animals, whereas suppression of antigen retention in other joint tissue could only be partly prevented. Although protamine equally affected antigen retention in the tissue of immune, as compared with nonimmune mice, the benefit of the protamine treatment was no longer detectable in the former group at Day 7. This was related to the fact that antigen in the tissue becomes cleared anyway, whether by lack of retention due to protamine-mediated competition or because of the vigorous inflammation-mediated antigen clearance.[4] Although not that impressive, some relative wiping out of the protamine effect on antigen retention in immune mice was also demonstrable at the cartilage level, probably due to loss of proteoglycans during inflammation and, therefore, loss of anionic binding sites. Nevertheless, suppression of joint inflammation by protamine was clear-cut at Day 3 and remained present at later stages (Figure 4 and Table 8).

The current view about the chronicity of joint inflammation in murine antigen-induced arthritis puts

Table 5 — Effect of Protamine on the Retention of $^{125}$I-aBSA in the Knee Joint of Nonimmune Mice

| First injection: protamine (mg/ml) | Second injection*: aBSA (mg/ml) | Antigen retention (%)† Day 1 | Day 2 | Day 4 | Day 7 |
|---|---|---|---|---|---|
| 10 | 1 | 95‡ | 92 | 103 | ND |
| 30 | 10 | 57 | 37 | 32 | 36 |
| 30 | 10§ | ND | 19 | 11 | 12 |
| 100 | 1 | 60 | 35 | 21 | 15 |
| 100 | 10 | 40 | 25 | 19 | 20 |

*Given 2 hours later.

†Expressed as the percentage of aBSA retained in protamine-pretreated knee joints compared with OA-pretreated joints.

‡Values represent the mean of groups of at least 5 mice.

§Simultaneous injection of protamine and aBSA.

Table 7—Effect of Protamine on the Retention of aBSA in Cartilage and Tissue

| | | Antigen retention (cpm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Patellar cartilage† | | | Tissue‡ | | |
| Day | Imm* | Protamine | OA | Prot/OA | Protamine | OA | Prot/OA |
| 2 | + | 28 ± 16 | 802 ± 238 | 4% | 273 ± 81 | 819 ± 288 | 33% |
| 4 | + | 33 ± 16 | 318 ± 114 | 11% | 174 ± 83 | 390 ± 178 | 45% |
| 7 | + | 20 ± 8 | 112 ± 54 | 18% | 65 ± 32 | 57 ± 30 | 114% |
| 2 | − | 40 ± 10 | 2335 ± 345 | 2% | 242 ± 62 | 1804 ± 956 | 13% |
| 7 | − | 14 ± 8 | 720 ± 56 | 2% | 36 ± 6 | 400 ± 98 | 9% |

Patellae with surrounding tissue were isolated at various days after intraarticular injection of 60 μg $^{125}$I-aBSA (700,000 cpm).
*Mice were immunized with aBSA/FCA (see Materials and Methods).
†Counts per minute retained in the cartilage of the whole patella.
‡Counts per minute retained per milligram of surrounding tissue of the patella.

emphasis on the role of retained antigen in the joint. Earlier studies in the rabbit made it clear that long-term retention of antigen by antibody-mediated trapping takes place in the avascular collagenous tissues of the joint.[2,3] Recent autoradiographic work from our laboratory in the murine arthritis model revealed that high amounts of the cationic antigen can be found in the loose connective tissues as well as in the cartilage shortly after intraarticular injection, but that antigen retention at later stages is almost exclusively found in the cartilaginous tissues.[5,14] It seems conceivable that antigen retained in the tissue plays an important role at the onset of joint inflammation, whereas antigen leaking from depots in the articular cartilage is more important at later stages. The exact time period by which the contribution of the cartilage depot becomes relevant remains, however, questionable.

It seems reasonable to assume that the effect of protamine on antigen retention in the tissue is high enough to be responsible for the observed suppression of joint inflammation at Day 3. Whether the suppression of arthritis found at later days (7–28) is the result of this early suppression or relates to the absence of an antigen depot in the cartilage, or both, remains to be seen.

Apart from suppression of joint inflammation, protamine treatment also caused a highly significant suppression of cartilage damage and bone apposition. Ultimate cartilage damage in this murine arthritis model correlates with both the severity and the chronicity of the joint inflammation.[15] The reduction seen in the chondrocyte death score (Table 8) may be the result of suppressed inflammation. In addition, total suppression by protamine of antigen (immune complex) depot formation in the cartilage may also contribute. A direct role of retained immune complexes in cartilage destruction has recently been suggested.[20,21] The fact that some chondrocyte death could be found in the protamine-treated mice indicates that retained immune complexes are not a pre-

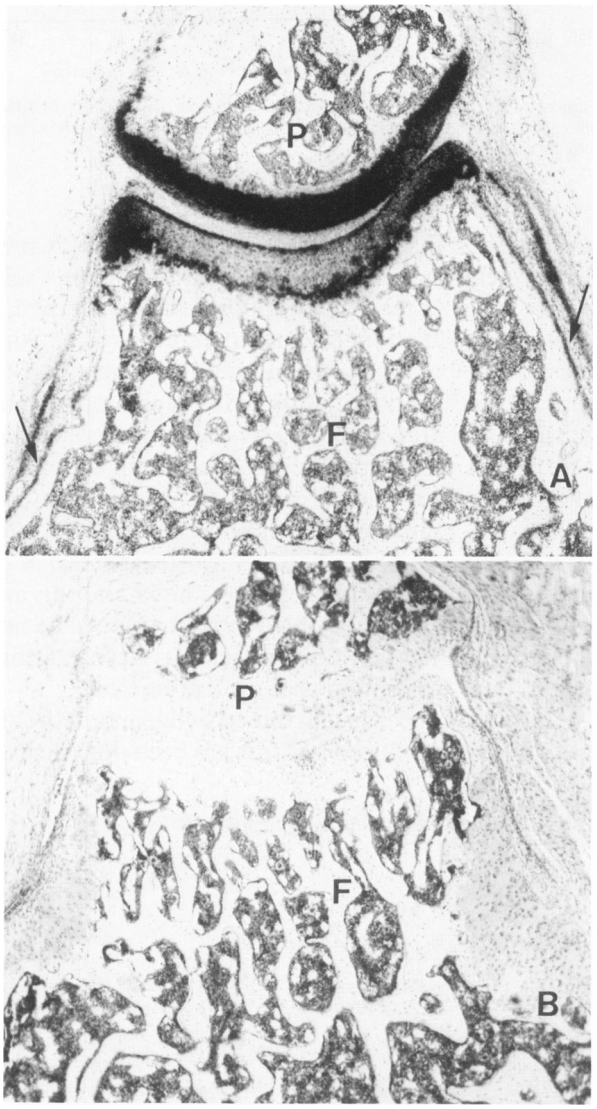


**Figure 3**—Autoradiographs of joint sections (×50) of nonimmunized mice at Day 7 after intraarticular injection of 60 μg $^{125}$I-aBSA together with a three times higher dose of OA (**A**) or protamine (**B**). Note the clear labeling of the cartilage and the presence of aBSA in the synovium and the periosteum (*arrows*) in the control and the absence of detectable labeling in the protamine-treated joint. Some synovial labeling could be seen in the latter joint at higher magnification. Further, note the absence of joint inflammation. *P*, patella; *F*, femur.

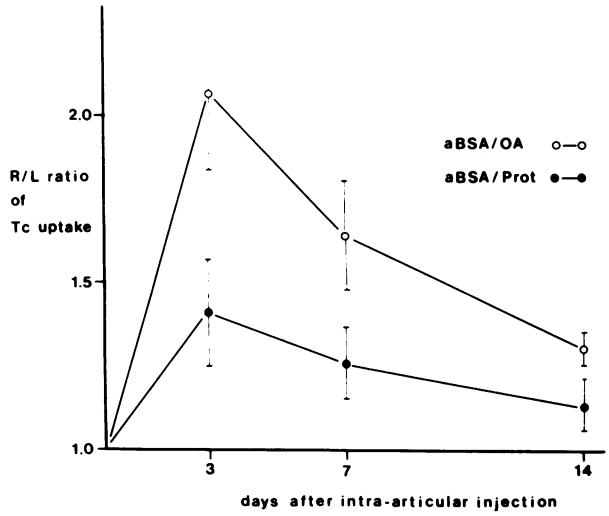


**Figure 4**—R/L ratios of $^{99m}$Tc uptake at various days after injection of aBSA with a three times higher dose of OA or protamine in aBSA/FCA-immunized mice. Values are the mean ± SD.

requisite and that, in any case, immune complex-mediated cartilage destruction is not the sole mechanism. As far as bone apposition is concerned, a twofold explanation also seems conceivable. Suppression of bone apposition could be due either to suppression of inflammation or to prevention of antigen accumulation in the epiphyseal growth plates and periosteum. Bone apposition occurs from activation of these compartments,[16,22] probably mediated by interleukin-1. Entrance of cationic antigen in the growth plates and retention in the osteogenic layer of the periosteum has been demonstrated,[5] and this antigen localization is prevented by protamine (data not shown). Whether this local antigen plays a pathogenic role in bone apposition is at present unclear.

Apart from the role of retained antigen, the chronicity of antigen-induced arthritis has been described to depend on adequate delayed hypersensitivity against the retained antigen.[1,2] Using mBSA as inducing antigen, Brackertz demonstrated the lack of induction of chronic arthritis in CBA mice, and this was correlated with the absence of adequate delayed skin test reactivity in this strain.[23,24] We could not find differences in mBSA retention in the joints of C57Bl, BALB/c, and CBA mice (unpublished observations), which indicated that the strain differences observed by Brackertz were solely related to T-cell reactivity. An unexplained finding was the lack of clear-cut haplotype dependence. Chronicity could be induced in $C_3H$ but not in CBA mice, both strains of the k/k haplotype.[23,24] With aBSA as inducing antigen, this study showed that chronic aBSA arthritis could be elicited in various strains. Although the severity and destruction appeared to be less in BALB/c mice, a lack of chronicity was certainly not found. Definite statements about sensitivity of strains is, however, not allowed with the present material, because in our hands some variation in severity of arthritis does occur upon immunization of one strain (C57Bl) on various occasions (unpublished data), and repeated experiments with large numbers of mice are needed to clarify this issue. In any case, a subtle relation with either antigen retention or delayed hypersensitivity was not apparent from our study.

Highly cationic proteins may potentially exert toxic effects, eg, cationic proteins released from activated eosinophils like major basic protein and eosinophil cationic protein. Adverse effects of protamine in the form of induction of joint inflammation were seen upon intraarticular injection of 6 $\mu$l of a high dose (100 mg/ml), but with the lower dosages used in this study unwanted side effects were not observed. Chondrocyte synthetic function was not affected by protamine injection, and *in vitro* experiments using a wide range of protamine concentrations indicated that in-

Table 8—Effect of Protamine on aBSA Arthritis

| Concomitant injection | n | Day | Histologic score* | | | |
|---|---|---|---|---|---|---|
| | | | Inflammation | | Joint damage | |
| | | | Infiltrate | Exudate | Chondrocyte death | Bone apposition |
| OA | 9 | 21 | 1.1 | 0.8 | 1.4 | 1.6 |
| Protamine | 10 | 21 | 0.5† | 0.2‡ | 0.5‡ | 0.5† |
| OA | 5 | 14 | 1.7 | 1.3 | 1.1 | 2.0 |
| Protamine | 5 | 14 | 0.9‡ | 0.5† | 0.2† | 0.6† |
| OA | 9 | 28 | 1.1 | 0.8 | 1.4 | 1.3 |
| Protamine | 10 | 28 | 0.4‡ | 0.2‡ | 0.5† | 0.5† |

Joints of immunized mice (aBSA/FCA) were given either protamine or OA (30 mg/ml) and aBSA (10 mg/ml). Arthritis was scored at Day 21 (first experiment) or at Days 14 and 28 (second experiment).

*Mean values scored on a three-point scale (see Materials and Methods).

†$P < 0.01$ (Mann–Whitney).

‡$P < 0.025$ (Mann–Whitney).

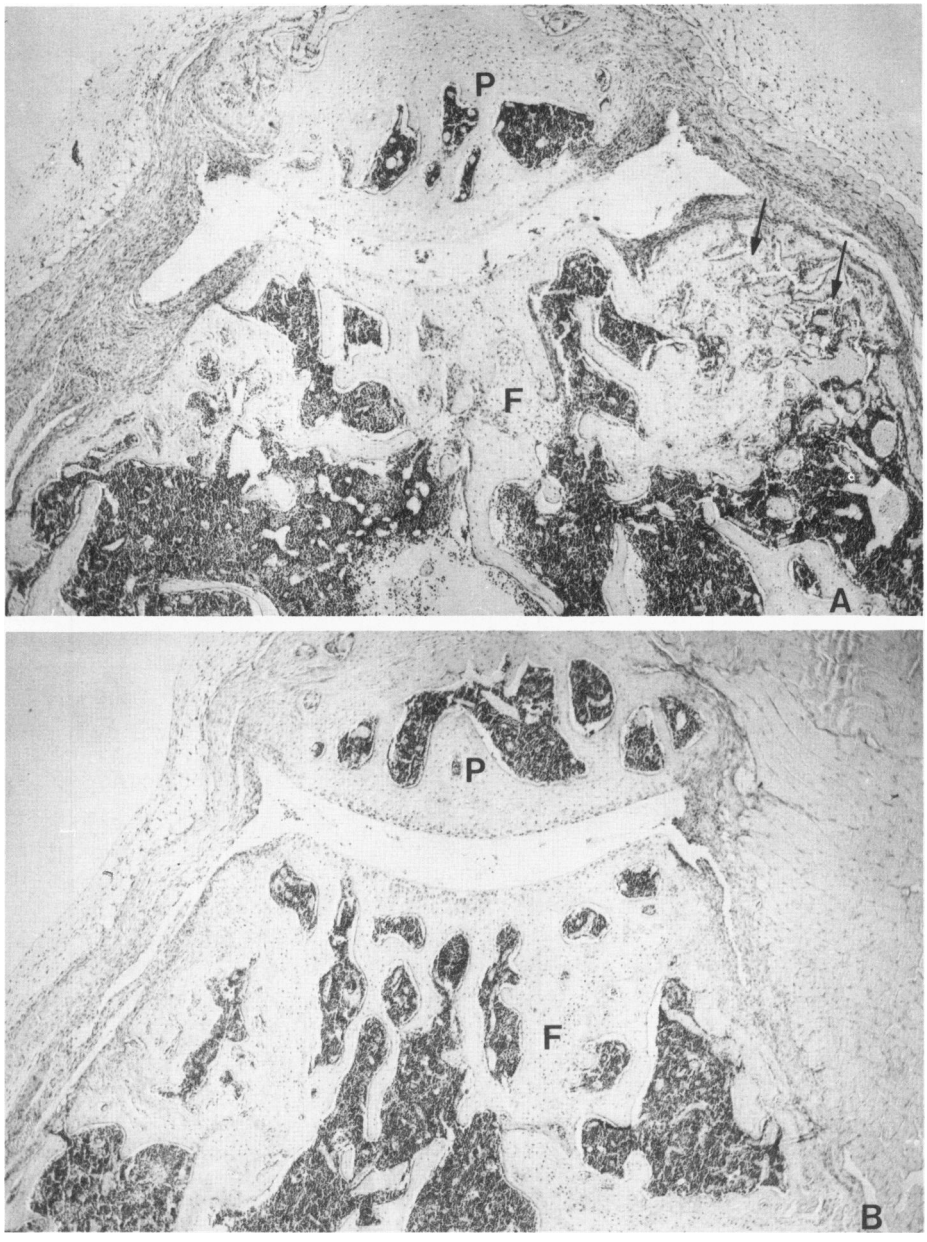


**Figure 5**—Representative histologic pictures (×50) at Day 21 after intraartricular injection of antigen in the aBSA/OA group (**A**) and the aBSA/protamine group (**B**) from Table 8. Note the chronic inflammation with cartilage destruction and new bone formation (*arrows*) in the control and minimal signs of arthritis in the protamine-treated joint. *P*, patella; *F*, femur.

hibition of chondrocyte function just occurred at high protamine concentrations in the cartilage, which were not reached in the present study (data not shown).

Treatment of arthritis has been approached by immunosuppression and anti-inflammatory therapy. The recent finding that experimenal membranous nephropathy could be prevented by nonimmunologic interference with *in situ* immune complex formation

Table 9—Effect of Protamine on Arthritis-Induced With Zymosan

| Injection* | R/L ratio of $^{99m}$Tc uptake | |
|---|---|---|
| | Day 3 | Day 7 |
| Zymosan + OA | 1.47 ± 0.09† | 1.10 ± 0.05 |
| Zymosan + aBSA | 1.34 ± 0.12 | 1.12 ± 0.07 |
| Zymosan + protamine | 1.67 ± 0.16 | 1.17 ± 0.09 |

*Concomitant intraarticular injection (6 μl) of zymosan (30 mg/ml) with OA, aBSA, or protamine (30 mg/ml).
†Values represent the mean + SD of groups of 5 mice.

Table 10—Effect of Protamine on a Delayed Hypersensitivity Reaction

| Injection* | Increase in ear thickness† | |
|---|---|---|
| | 24 hours | 48 hours |
| mBSA + OA | 23 ± 2‡ | 31 ± 4 |
| mBSA + protamine | 24 ± 3 | 29 ± 4 |

*Five micrograms mBSA combined with either 15 μg OA or protamine.
†Skin test in the ear at Day 21 after immunization of mice with mBSA/FCA.
‡Values represent the mean + SD of groups of 5 mice.

using the competitive polycation protamine[9] has prompted us to study its beneficial effects in arthritis. Like its role in the nephritis model,[7,8] electrical charge was also shown to play an important role in chronic murine arthritis.[4,5] This is the first report to show that competition with a competitive cationic protein can be used as therapy to prevent local antigen retention in the joint and to suppress joint inflammation. Exacerbations of murine joint inflammation are described upon systemic administration of antigen.[25,26] It is hoped that such flare-up reactions, which are caused by antigen deposition in the joint,[27] could likewise be prevented with competitive substances. Further studies are needed with the use of various polycations and interference at various phases of joint inflammation to elucidate the usefulness of this approach in the treatment of arthritis.

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