Cationization of Catalase, Peroxidase, and Superoxide Dismutase

Effect of Improved Intraarticular Retention on Experimental Arthritis in Mice

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Abstract

Several enzymes and other proteins were made cationic either by coupling to polylysine or by shielding of anionic sites. These cationic proteins, all having an isoelectric point >8.5 exhibited excellent retention in articular structures when injected in mouse knee joints. Autoradiography and histochemistry showed that cationic forms of catalase, superoxide dismutase, and horseradish peroxidase were firmly retained by synovial and cartilaginous tissues. The half-life of these enzymes in the joint is thus significantly extended compared with native enzymes. The native enzymes and their cationic derivatives were tested for antiinflammatory properties in mice, using antigen-induced arthritis and zymosan-induced arthritis. It was found that injection of cationic catalase or peroxidase induced a marked suppression of some parameters of the inflammatory response in both types of arthritis, as measured by ^{99m}technetium pertechnetate uptake and leakage of ^{125}I-labeled albumin. Native catalase and peroxidase were less, or not at all effective. Cationic superoxide dismutase or cationic nonenzyme proteins did not suppress inflammation. The observed suppression of two different types of inflammation (an immune and a nonimmune arthritis) by catalase and peroxidase suggests that elimination of peroxides contributes to the suppression of an inflammatory response.

We would hypothesize that cationic enzymes offer the possibility for investigating the mechanisms of inflammation and, in addition, might be interesting from a therapeutical point of view.

Introduction

Since it has been established that activated neutrophils produce vast amounts of reactive oxygen metabolites (1, 2), a still growing body of evidence indicates that these oxygen-derived metabolites play an important role in inflammatory response and concomitant tissue damage. Most of the indications for the involvement of oxygen-derived metabolites in tissue damage stem from in vitro experiments. There are several reports on free radical-mediated degradation of macromolecules (3–5) and damage to cultured cells as a result of exposure to activated inflammatory cells or superoxide-producing systems (6–9). Recently we showed that the chondrocyte proteoglycan synthesis is suppressed by hydrogen peroxide (10, 11). Since it was found in vitro that several enzymes provided protection against free radical-induced damage, it was hoped that these enzymes could be used as antiinflammatory drugs. In several experimental models of inflammation, an effect of administered superoxide dismutase (SOD)[1] or catalase was observed (12–15). In some of these models the hydroxyl radical was proposed as the toxic species, a possibility supported by the observations that ionic iron potentiates the inflammation and iron chelators are inhibitory (16, 17).

One of the problems with therapeutic use of enzymes is their limited half-life in the body. SOD, which is sold commercially and is reported to be effective in humans, has a half-life in serum of 5–7 min (13). It is readily accumulated in the kidneys and excreted in the urine. The chance that a significant amount of intramuscularly administered SOD reaches an inflammatory focus seems remote. It has been shown that a prolonged half-life of SOD (by coupling to Ficoll) (18) potentiates its antiinflammatory action.

Most of the work on enzymes as antiinflammatory drugs was performed with SOD. Occasionally catalase is reported to be effective (14, 15). In the present study we used several enzymes (SOD, catalase, and horseradish peroxidase [HRPO]) modified to yield an isoelectric point (IEP) >8.5. Cationic proteins exhibit a strong affinity for negative articular structures when injected in mouse knee joints (19, 20). Thus, the half-lives of these cationic enzymes in the joint were improved significantly compared with the native enzymes. Cationic derivatives of catalase and peroxidase were found to be very effective in suppressing two different types of experimental arthritis. Cationic SOD was not effective. Our data suggest that peroxides (hydrogen peroxide, lipid peroxides) play an important role in the inflammatory response.

Methods

Animals. Male 7–9-wk-old C57 black mice weighing 22–26 g at the start of the experiment were used. They were fed a standard diet and tap water ad lib.

Materials. Catalase (thymol-free, 17,600 U/mg), SOD (2,800 U/mg), HRPO (275 U/mg), xanthine oxidase (1.3 U/mg), xanthine (grade V), cytochrome *c* (type IV), methylated bovine serum albumin (mBSA), Zymosan A, ovalbumin (OA), poly L-lysine (30,000–70,000 mol wt), 3,3′-diaminobenzidine hydrochloride (DAB), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I (carrier-free) was purchased from Amersham

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1. *Abbreviations used in this paper:* a, amidated; AIA, antigen-induced arthritis; DAB, 3,3′diaminobenzidine hydrochloride; DMPA, *N,N*-dimethyl-1,3-propanediamine; H&E, hematoxyline and eosine; HRPO, horseradish peroxidase; IEP, isoelectric point; mBSA, methylated BSA; MSA, murine serum albumin; OA, ovalbumin; PLP, polylysine coupled to HRPO; PLP_{inact}, heat-inactivated PLP; PAGE, polyacrylamide gel electrophoresis; SOD, superoxide dismutase; ^{99m}Tc, ^{99m}technetium pertechnetate; ZIA, zymosan-induced arthritis.

(Bucks, England). K-5 Photographic emulsion was obtained from Ilford Ltd. (Basildon, Essex, England). *N,N*-Dimethyl-1,3-propanediamine (DMPA) was obtained from BDH Chemicals Ltd. (Poole, England). Hydroxyphenylpropionic acid was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England). Isoelectric focusing was performed on a precast gel from Pharmacia Fine Chemicals (Uppsala, Sweden).

Enzyme modification. Catalase, SOD, BSA, and OA were modified according to the method of Danon et al. (21), using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as an activator and DMPA as a nucleophile as described previously (20). In this way free carboxyl groups of the protein were coupled to amino groups of DMPA. The IEP of the protein was thus raised, since anionic groups were eliminated and cationic groups were introduced by DMPA. We refer to this procedure as "amidation" of the protein. Thus, proteins modified as described above have the prefix "a" (e.g., aCatalase, amidated catalase).

HRPO was made cationic by coupling to polylysine. Briefly, the sugar moieties of HRPO were oxidized to the corresponding aldehyde with $NaIO_4$, and coupling to poly L-lysine was performed at pH 9.5.

Enzyme measurements. Peroxidase was assayed fluorimetrically, using a modification of the procedure described by Zaitsu and Ohkura (22) based on the fluorescence of the oxidation product of hydroxyphenylpropionic acid (λ_{ex} = 325 nm, λ_{em} = 405 nm).

SOD was assayed by its ability to inhibit cytochrome *c* reduction by superoxide, which was generated by xanthine-xanthine oxidase (23). Catalase was assayed at pH 7.0 by the fall in absorbance (240 nm) from 0.45 to 0.40 as H_2O_2 was destroyed (24).

Iodination of enzymes. ^{125}I-Labeling was performed by the chloramine T method (25). ^{125}I-Protein was separated from free ^{125}I by Sephadex G-25 fractionation.

Determination of IEP. The IEP of the various proteins was determined by isoelectric focusing on a 5% polyacrylamide slab gel, using an ampholyte pH gradient from 3.5 to 9.5, according to the manufacturer's instructions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated on an 8–16% polyacrylamide gradient slab gel according to the method of Laemmli (26).

Immunization and induction of arthritis. Mice were immunized with 100 μg mBSA in 0.1 ml Freund's complete adjuvant emulsion as previously described (27). On day 21 after the primary immunization, arthritis was induced by the intraarticular injection of variable doses of antigen in 6 μl of saline in the right knee joint. The nonimmune arthritis was induced by the intraarticular injection of zymosan in 6 μl of saline, as previously described (27).

Experimental design. Generally, six to nine mice per group were treated with enzyme 1 d before the induction of arthritis. 6 μl of enzyme solution was injected into the right knee joint. After induction of arthritis (as described above) the inflammation was quantitated with ^{99m}technetium pertechnetate (^{99m}Tc) uptake, usually at day 3 and 7 after induction.

Measurement of arthritis. To quantify the joint inflammation we used an adaptation of the ^{99m}Tc uptake (28) as described previously (29, 30). Briefly, mice were injected with 10 μCi ^{99m}Tc and sedated with chloral hydrate. After 30 min the amount of radioactivity in the right and left knee joint was assessed by measuring the γ-radiation, with the knee in a fixed position, using a collimated NaI scintillation crystal. Arthritis was scored as the ratio of the ^{99m}Tc uptake in the right and left knee joint. Right/left ratios > 1.10 were taken to indicate inflammation of the right knee joint.

Measurement of enzyme retention. The retention of cationic and native enzymes in vivo was assessed using radiolabeled (^{125}I) enzymes. After intraarticular injection of 12 μg of enzyme (6 μCi/mg), the retained enzyme was quantitated by external gamma counting, as described for measurement of ^{99m}Tc.

Quantification of vascular permeability. Vascular permeability was quantitated by the degree of extravasation of ^{125}I-labeled murine serum albumin (MSA). This method is similar to assays that have proved to be useful in lung and dermal inflammation (14, 31). ^{125}I-Labeled MSA was injected intravenously (4 μCi/mouse), and after 1 h the accumulation of radiolabel in the knee joints was measured, using the device described for ^{99m}Tc measurement. The ratio of the measured radioactivity in the right (inflamed) and left (control) knee joint was taken as a measure for the plasma exudation as a result of the inflammatory response.

Histology and autoradiography. Mice were killed by ether anaesthesia. The knee joints were dissected and processed for histology as previously described (27). Total knee sections (6 μm) were prepared, mounted on gelatin-coated slides and stained with hematoxyline and eosine (H&E). Paraffin sections of the total knee were dipped in photographic emulsion and exposed for 5–20 d. After this period the slides were developed and stained with H&E.

Histochemistry. For demonstration of peroxidase activity in articular tissues cryostat sections of whole undecalcified knee joints were obtained using a method recently developed in our laboratory (32). After fixation in 4% buffered formaline (2 min at room temperature) the sections were incubated in Tris buffer pH 7.8 with 0.05% DAB and 0.2% hydrogen peroxide for 20 min at room temperature. The rinsed sections were embedded in 50% glycerol.

Statistics. The quantitation of arthritis with ^{99m}Tc uptake or ^{125}I-MSA leakage was evaluated statistically using the one-tailed Mann-Whitney U test. Significance was calculated for the enzyme-treated groups compared with the appropriate controls: aOA for aCatalase and aSOD, heat-inactivated PLP (PLP_{inact}) for polylysine-coupled HRPO (PLP).

Results

Enzyme modification. SOD, catalase, BSA, and OA were amidated as described. HRPO was coupled to poly L-lysine; this procedure could not be applied to the other proteins since it relies on the presence of oxidizable sugar moieties. Fig. 1 shows an isoelectric focusing gel of native and modified proteins. Due to the relatively mild conditions of protein modification that avoid extreme pH and temperature, there was little loss of enzyme activity (aSOD, 80–90%; aCatalase, 100%; and PLP, 100% of the original activity is retained; enzymes assayed as described in Methods). Amidation caused chemical modification of the enzymes but their molecular weights were not significantly altered. Inter- and intramolecular

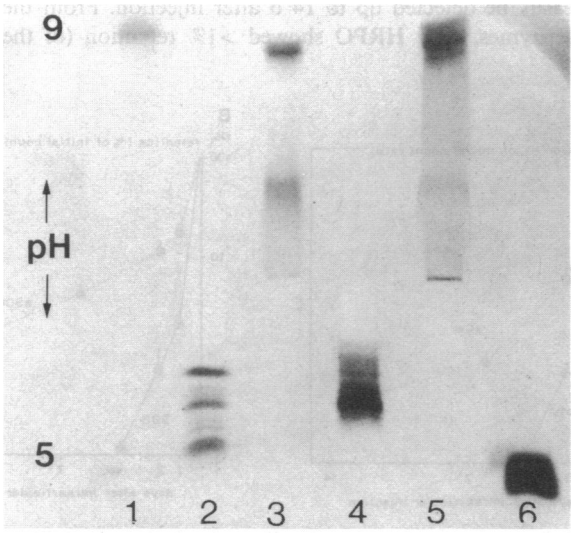

Figure 1. Isoelectric focusing slab gel (pH gradient 3.0–9.5) of native and cationic proteins. Lanes 1–6: aSOD, SOD, aCatalase, catalase, aBSA, and BSA, respectively.

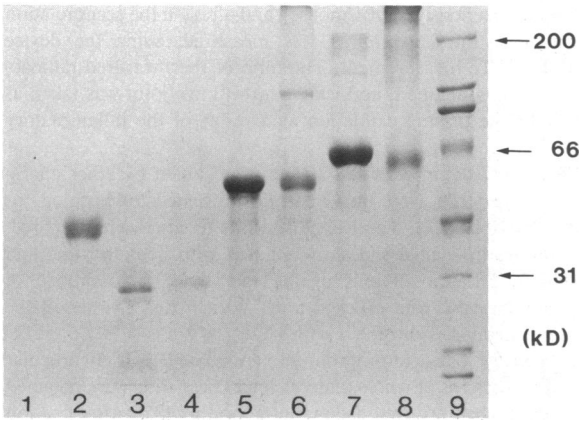

Figure 2. SDS-PAGE of native and cationic proteins (8–16% gradient slab gel). Lanes 1–9: PLP, HRPO, SOD, aSOD, catalase, aCatalase, BSA, aBSA, and molecular weight markers, respectively. Molecular weights indicated in kilodaltons. Lane 1 appears to be empty because PLP consists of large molecules that do not enter the gel.

cross-linking may have occurred to some extent as can be seen on Fig. 2. Apart from the catalase monomer (60 kD), small amounts of dimer and trimer are visible. Since this gel was run under dissociative conditions, some covalent coupling of the monomers must have occurred as a result of the amidation. Coupling of HRPO to polylysine caused less chemical modification, but the molecular weight was significantly raised since several polylysine molecules were coupled to HRPO. SDS-PAGE (Fig. 2) of the modified enzymes showed that PLP is a complex of >200 kD that does not enter the gel.

Retention studies. To investigate the effect of charge modification on the retention of the enzymes, the clearance of native and cationic radiolabeled enzymes was measured after intraarticular injection in mouse knee joints. 12 μg of enzyme (6 μCi ^{125}I/mg protein) in saline was injected and retention was measured by external gamma counting. Fig. 3 shows that retention of the cationic enzymes was considerably improved compared with the unmodified enzymes. Cationic enzymes could easily be detected up to 14 d after injection. From the native enzymes, only HRPO showed >1% retention (of the initial dose) at day 2, which can be attributed to the presence of basic isoenzymes. The cationic derivatives were significantly better retained: 7–14% of the initial dose on day 2. The bulk of the injected enzyme was rapidly cleared but the amount of enzyme retained after day 2 exhibited an extremely long half-life.

Localization of cationic enzymes. To confirm the quantitative data on enzyme retention and to investigate the distribution of radiolabeled cationic enzymes within the joint, whole joint sections were autoradiographed. Native SOD, catalase, and HRPO were poorly retained (quantitatively) and localized predominantly in the synovium and to a lesser extent on fibrocartilage; no affinity for hyaline cartilage was observed. The cationic derivatives all showed a strong affinity to cartilaginous and synovial structures as visualized with autoradiography. aCatalase and aSOD were predominantly associated with hyaline and fibrous cartilage, whereas PLP was retained both on cartilage and in the synovium as visualized in Figs. 4 and 5.

Histochemistry. To check whether the retained radioactivity represented active enzyme, we performed histochemistry on cryostat sections of knee joints at various times after intraarticular injection of PLP. aSOD and aCatalase were not studied since no histochemical detection is available for these enzymes. Active enzyme was demonstrable at least up to 7 d after injection (Fig. 5).

Effects on experimental arthritis. Two models of experimental arthritis in mice, antigen-induced arthritis (AIA) (33) and zymosan-induced arthritis (ZIA) (34), were used. The first is a T lymphocyte-dependent inflammation (33), the latter is, at least in the acute phase, not driven by immunological mechanisms. Both types of inflammation have a protracted course due to persistence of the irritant. aCatalase was tested in an AIA (Table I). 1 d before induction of arthritis with 40 μg of mBSA, mice were treated with aCatalase, catalase, aOA, or saline. aOA served as a control; this protein is cationic but does not possess enzymatic activity. We could not use heat-inactivated aCatalase since inactivation always causes precipitation of this enzyme; thus it would not represent a proper control. Joint inflammation was measured at 3 and 7 d after induction. Table I suggests a moderate (not significant) effect of both aCatalase and catalase after 3 d. However, after 7 d,

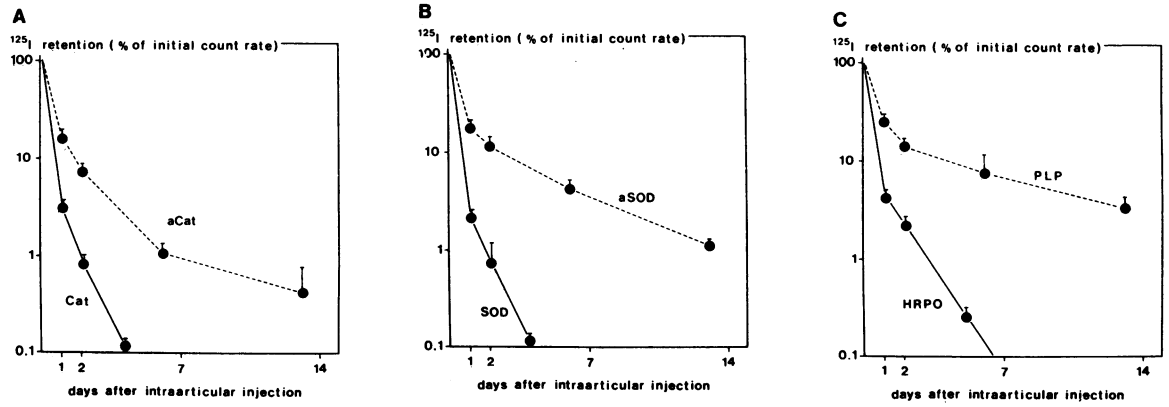

Figure 3. Retention curves of (*A*) aCatalase, (*B*) aSOD, and (*C*) PLP. 12 μg of radiolabeled cationic or native enzyme was injected. The amount of injected radioactivity was considered as the initial 100% value. The retention of radiolabeled enzyme was monitored by external γ-counting and expressed as a percentage of the initial dose. Each point represents the average of five kneejoints±SD.

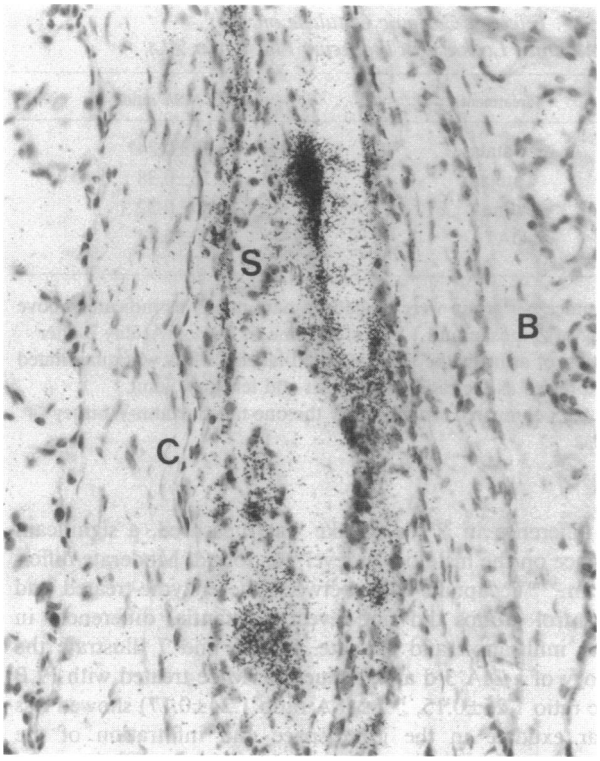

Figure 4. Autoradiograph of a mouse knee joint 14 d after intraarticular injection of ^{125}I-labeled PLP. Note the retention of cationic enzyme in synovial tissue. H&E staining. Original magnification × 250. S, Synovium; C, capsule; and B, bone.

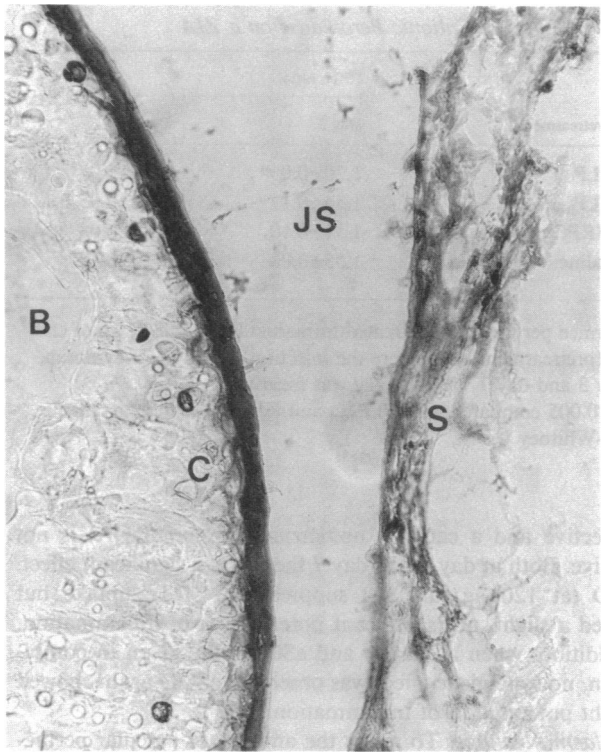

Figure 5. Histochemical demonstration of peroxidase activity in a cryostat section of an undecalcified knee joint 5 d after intraarticular injection of 12 μg of PLP. Notice the dark precipitates of the DAB reaction on the cartilage surface and in the synovial tissue. No counterstaining used. Original magnification × 250. JS, Joint space; B, bone; C, cartilage; and S, synovium.

inflammation as measured with ^{99m}Tc uptake had waned in the aCatalase group, but arthritis was still clearly present in the other groups, including the mice that had received native catalase. Since inflammation is related to the amount of antigen retained in the joint, we checked whether treatment with cationic proteins before the induction of arthritis had any effect on mBSA retention. We did not detect any effect of the pretreatment (with cationic enzymes) on the retention of ^{125}I-labeled mBSA. In addition, aOA did not suppress the inflammation (Table I); it was therefore concluded that the enzymatic activity of aCatalase was responsible for the observed effect. In a separate experiment, SOD and aSOD were tested for effects in the acute and late phase of inflammation in AIA (day 3 and day 7); however, no antiinflammatory effects of native or aSOD were observed (data not shown).

PLP was tested in another model of arthritis (Table II) together with the native enzyme. Since PLP can be inactivated by heating (10 min, 100°C) without precipitation, this preparation represents an adequate control for the active cationic enzyme. In this experiment 180 μg of zymosan was injected intraarticularly 1 d after intraarticular injection of PLP, PLP_{inact}, HRPO, and saline, respectively. At day 3 and 7 arthritis was measured with ^{99m}Tc uptake. Table II shows that PLP had a significant effect on the inflammatory response compared with PLP_{inact}, HRPO, and saline. In other experiments we tested PLP in an AIA and found essentially the same results as for the ZIA. We also varied the administration schedule for the cationic enzymes. Basically similar results were obtained whether the enzymes were given 3 d before the induction of arthritis or on the same day (mixed with the inflammatory agent). The effect of aCatalase was found to be dose-dependent. We observed that the effect reached a plateau at doses from 12 to 120 μg of enzyme.

Table III shows the effects of aCatalase and aSOD in a ZIA. In this experiment we also tested whether aSOD would enhance the effect of aCatalase. We also showed the effects of very high doses of enzyme (120 μg of protein). This experiment shows results similar to those obtained with the AIA. aCatalase

Table I. Effect of Cationic Catalase on an AIA

	^{99m}Tc ratio	
Pretreatment	Day 3	Day 7
aCatalase	1.36±0.20	1.06±0.08*
Catalase	1.42±0.12	1.36±0.10
aOA	1.50±0.27	1.35±0.12
Saline	1.62±0.26	1.41±0.09

Eight mice per group (immunized with mBSA) were injected intraarticularly with 12 μg of protein (pretreatment) 1 d before the injection of 40 μg of mBSA in the same knee joint. At day 3 and 7 after induction of arthritis the inflammation was quantitated with ^{99m}Tc uptake.

* $P < 0.005$ compared with aOA and catalase by the one-tailed Mann-Whitney U test.

Table II. Effect of Cationic Peroxidase on a ZIA

Pretreatment	^{99m}Tc ratio	
	Day 3	Day 7
PLP	1.20±0.05*	1.03±0.05*
PLP_{inact}	1.54±0.17	1.19±0.05
HRPO	1.51±0.10	1.23±0.08
Saline + zymosan	1.55±0.08	1.26±0.10

Eight mice per group were treated intraarticularly with 12 μg of enzyme (pretreatment) 1 d before the injection of 180 μg of zymosan. At day 3 and day 7 ^{99m}Tc uptake was measured.
* $P < 0.005$ compared with PLP_{inact} and HRPO by the one-tailed Mann-Whitney U test.

Table IV. Effect of Cationic Catalase on [^{125}I]Albumin Leakage in the Acute Phase of a ZIA

Treatment	^{125}I-MSA ratio
aCatalase	1.73±0.24*
Catalase	2.13±0.38
Saline	2.49±0.32
aOA	2.20±0.44

Seven mice per group were treated intraarticularly as indicated above (240 μg of zymosan and 12 μg of enzyme in saline). At day 2 after induction of arthritis the leakage of ^{125}I-labeled MSA was quantitated and expressed as the ratio of the right and left knee joint.
* $P < 0.05$ compared with aOA by the one-tailed Mann-Whitney U test.

is effective and a cationic nonenzyme protein (aOA) is not effective. Both at day 3 and day 7 there was a significant effect. aSOD (at 120 μg) did not suppress the ^{99m}Tc uptake but showed a slight, not significant potentiation of inflammation. In addition, when aCatalase and aSOD were given in combination, no synergistic effect was observed; aSOD again showed a slight potentiation of inflammation.

Plasma leakage. To assess the amount of vascular permeability or vascular damage as a result of the inflammatory response, we measured the extravasation of ^{125}I-labeled MSA. Table IV shows that aCatalase significantly decreases the leakage of plasma protein at day 2 of a ZIA compared with catalase and aOA. Similar results were obtained for PLP in a ZIA. No significant effect was observed with HRPO.

Morphologic analysis. Inflammation of enzyme-treated and untreated animals was examined macroscopically when the knee joints were dissected for histology. In general it was observed that mice treated with aCatalase or PLP in either type of inflammation showed markedly less swelling and periarticular bleeding. Even when the differences in ^{99m}Tc uptake were relatively mild, macroscopical differences between the enzyme-treated and the control groups were evident. Light microscopic examination of arthritis showed that only when a large difference in ^{99m}Tc uptake was measured, a significant difference on the histological level was found. Moderate differences in ^{99m}Tc uptake ratio between the enzyme-treated and the control groups did not reveal substantial differences in cellular infiltration and exudate. Figs. 6 and 7 illustrate the histology of a ZIA 3 d after induction. Mice treated with PLP (^{99m}Tc ratio 1.25±0.15, ^{125}I-MSA ratio 1.24±0.17) showed less cellular exudate in the joint space and infiltration of the synovium than animals treated with PLP_{inact} (^{99m}Tc ratio 1.70±0.34, ^{125}I-MSA ratio 1.91±0.52).

Table III. Effect of Cationic Catalase and SOD on a ZIA

Treatment	^{99m}Tc ratio	
	Day 3	Day 7
aCatalase (12 μg)	1.29±0.09*	1.10±0.05*
aCatalase (120 μg)	1.28±0.09*	1.14±0.04*
aSOD (12 μg)	1.86±0.14	1.54±0.10
aSOD (120 μg)	1.97±0.12	1.62±0.12
aOA (12 μg)	1.82±0.14	1.58±0.15
aOA (120 μg)	1.79±0.15	1.49±0.08
aCatalase (12 μg + aSOD 12 μg)	1.48±0.14*	1.21±0.09*
Saline	1.86±0.14	1.53±0.11

Seven mice per group were treated intraarticularly with 180 μg of zymosan and varying doses of enzyme. At day 3 and 7 after induction of arthritis ^{99m}Tc uptake was measured.
* $P < 0.005$ compared with aOA by the one-tailed Mann-Whitney U test.

Discussion

The data presented above basically show two distinct phenomena. Firstly, cationized proteins (aSOD, aCatalase, PLP) exhibited excellent retention in articular structures compared with the native proteins (SOD, catalase, HRPO). Secondly, application of cationic enzymes in experimental joint inflammation revealed that two enzymes capable of eliminating peroxides (PLP, aCatalase) were able to suppress the inflammatory response in two types of experimental arthritis. Suppression of

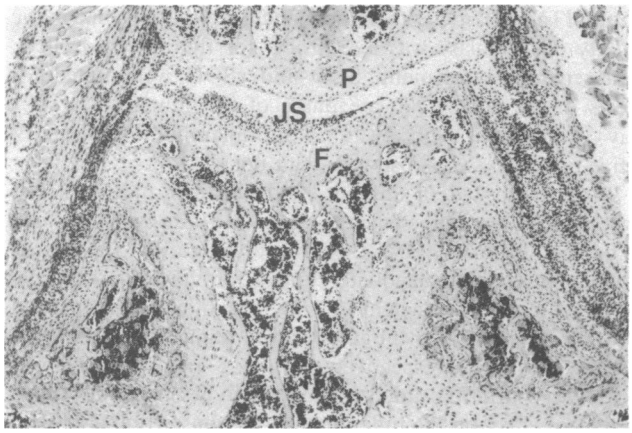

Figure 6. Section of a mouse knee joint at day 3 of a ZIA treated with PLP_{inact}. Large numbers of inflammatory cells (mainly neutrophils) are visible in the joint space, and the synovial tissue is heavily infiltrated (neutrophils and mononuclear cells). Original magnification × 100. H&E staining. P, Patella; F, femur; and JS, joint space.

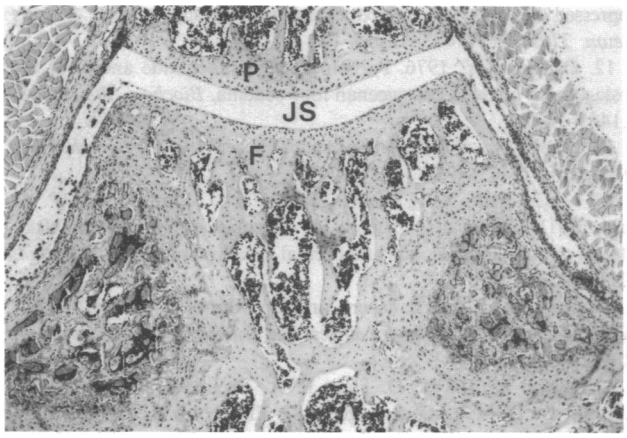

Figure 7. Section of a mouse knee joint at day 3 of a ZIA treated with PLP. A moderate amount of cellular exudate and infiltrate is visible. Original magnification × 100. H&E staining. P, Patella; F, femur; and JS, joint space.

arthritis was observed both in the acute and in the chronic phase, as measured with ^{99m}Tc uptake.

Recently attention has been focused on electrical charge of macromolecules as an important determinant for retention. Anionic sites on the glomerular basement membrane, articular structures, and cell surfaces have been shown to exhibit a strong affinity for cationic agents (19, 20, 35). Most of these studies are concerned with the retention and handling of cationic antigens. Several models using cationized antigens for the induction of experimental nephritis or arthritis have been described (33, 35, 36). The mechanism of retention is probably the interaction with negatively charged proteoglycans which are abundantly present in basal membranes and cartilaginous structures (and to a lesser extent in all connective tissue). We applied the retention potential of cationic proteins to several enzymes that are possible candidates for modulation of an inflammatory response. We used amidation of enzymes (20, 21) and coupling of a glycoprotein enzyme to a polycation. Our data show that these procedures resulted in a prolonged half-life of the enzymes in the joint. The native enzymes SOD and catalase are known to suppress the inflammatory response in several experimental models (12–15). The mechanisms of the suppressive action could be direct, by elimination of potentially toxic agents as superoxide and hydrogen peroxide, or indirect, by prevention of the formation of hydroxyl radicals and lipid peroxides. Although several investigators independently demonstrated the effects of scavenging enzymes in different models of experimental inflammation, other data cast some doubt on the general validity of these findings (37, 38).

One of the serious drawbacks of the application of enzymes for systemic or local (e.g., intraarticular) use is the rapid clearance of the enzyme. It is hard to imagine how intramuscularly administered SOD (as it is applied clinically) could reach an arthritic joint in sufficient amounts to exert an effect. The half-life in serum is reported to be 5–7 min (13, 18); in addition, SOD is not taken up by cells (13), indicating that transportation to the inflammatory focus by macrophages or neutrophils is not very likely. SOD coupled to Ficoll (18) has been tested in animal models and was found to be far more effective than the native enzyme. The half-life in serum was thus extended to several hours due to the increased molecular weight of the enzyme. In contrast to the data available on the application of SOD and catalase in experimental inflammation, reports on the effect of peroxidases are scarce. Surprisingly, we did not find any beneficial effect of SOD or aSOD in either type of inflammation. The cationic derivatives of catalase and HRPO were highly effective in two types of experimental arthritis. In addition, both in the acute and the chronic phase suppression of inflammation was found. In the acute phase of arthritis, sometimes a mild effect of the native enzymes was observed. We never saw any significant effect of the native enzymes after the acute phase. This observation is consistent with the retention data which indicate a large discrepancy in clearance rate after day 2. Nonenzyme proteins (aOA, aBSA) and PLP_{inact} did not suppress inflammation. Thus we conclude that the observed effect on arthritis was associated with peroxidase activity and not with an anomalous behaviour of cationic proteins per se. Apart from the surprising result that aSOD did not modulate the inflammation at all, it has to be noted that the impact of peroxidase and catalase was not limited to the acute phase of arthritis. The reported beneficial effects of SOD in experimental models are usually confined to a specific stage of the inflammatory response. For instance in the carrageenan-induced edema, SOD suppresses the "prostaglandin phase" of inflammation; it was, therefore, concluded that superoxide production potentiated the inflammation because it was linked with the arachidonic acid metabolism (12).

Our data indicate that the assessment of the effect of peroxidase enzymes depends on the use of the cationic derivatives. The effects of the native enzymes (in the acute phase) are too small to be measured, considering the variance inherent to experimental models of inflammation. The mechanism by which peroxidase or catalase exerted its effect on inflammation could be the elimination of hydrogen peroxides or lipid peroxides. The physiological function of catalase is its ability to prevent high intracellular concentrations of hydrogen peroxide. The affinity for hydrogen peroxide is rather low, and the catalytic activity of the enzyme is related to the concentration of the substrate (39). At low concentrations of hydrogen peroxide, catalase exhibited little catalytic activity but peroxidatic activity increased. Catalase cannot use organic peroxides (39) as a substrate in contrast with true peroxidases. HRPO is active as a peroxidase at low concentrations of hydrogen peroxide and exhibits a low specificity with respect to oxidizable substrates. Hydrogen peroxide is receiving increasingly more attention as a mediator of inflammation and tissue damage. It has been shown to be far more toxic to cells than superoxide. Cultured cells are killed by relatively low concentrations of hydrogen peroxide (7–9), and we have recently demonstrated suppression of chondrocyte proteoglycan synthesis in intact articular cartilage by hydrogen peroxide (10, 11). Recently it was shown that extremely low concentrations of hydrogen peroxide induce prostaglandin synthesis by endothelial cells (9). In view of these data, hydrogen peroxide seems a likely candidate to serve as an important substrate for the enzymes that we applied. Since lipid peroxides are known to be potent chemoattractants, and considering the relatively broad specificity of peroxidases, it is also conceivable that elimination of lipid peroxides accounts for the observed suppression of inflammation. Another possible explanation of the peroxidase

effects is the observation that peroxides in general (including hydrogen peroxide and lipid peroxides) are potent activators of the enzyme cyclooxygenase (40). Elevated levels of cyclooxygenase activity might also contribute to potentiation of the inflammatory response.

The effect of aCatalase and PLP seems to be suppression of the effector mechanisms of inflammatory cells rather than a suppression of the number of cells, since in the enzyme-treated animals large numbers of neutrophils were present. This phenomenon has recently been demonstrated in two other models of inflammation (41, 42). The effects of hydrogen peroxide on endothelial cells, demonstrated in vitro (9), are in accordance with our findings that indicate protection of vascular endothelium by aCatalase and PLP, since ^{99m}Tc uptake and ^{125}I-MSA leakage are suggestive for vascular damage.

We believe that the improved retention potential of inflammation-modulating enzymes will significantly contribute to elucidating certain aspects of the inflammatory response. In addition, these findings may encourage the study of the clinical use of enzymes as antiinflammatory drugs.

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