RHEUMATOID arthritis (RA) is characterized by an abnormal cellular and cytokine infiltration of inflamed joints. This study addresses a previously unrecognized interaction between neutrophilic-myeloperoxidase (MPO) and macrophages (Mø) which could explain the perpetuation of inflammation associated with RA. A monoarticular arthritis was induced in female Lewis rats by injection of streptococcal cell wall extracts (PG-APS). After swelling and erythema subsided, joints were re-injected with one of the following: porcine MPO or partially inactivated MPO (iMPO). Injection with either MPO or iMPO induced a 'flare' of experimental RA. Blocking the Mø-mannose receptor by mannans, ablated exacerbation of disease. These results indicate that MPO or iMPO can play a pivotal role in *the perpetuation but not initiation* of this RA model.

Perpetuation of inflammation associated with experimental arthritis: the role of macrophage activation by neutrophilic myeloperoxidase

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Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease of unknown etiology.[1–3] It is known that this inflammatory disease affects the synovial membranes of various joints and that there is *de novo* vascularization beneath the synovial lining along with an orchestrated infiltration of the joint by T and B lymphocytes as well as macrophages (Mø). Several investigators have reported the association of Mø with the pathology of RA.[4–7] Moreover, it has been reported that 80–100% of those cells which line the synovium are Mø.[8] In addition to the Mø, neutrophils are abundant in the synovial fluid and it has been reported that both cell types are found in a state of heightened activation.[2,3,8] It has recently been reported that Mø directly influence a neutrophil-dependent inflammatory response and that the neutrophil, in turn, recruits and activates Mø.[9]

Along with the presence of the above cell types, it is documented that there are abnormal levels of numerous cytokines in an RA joint.[1,8] Among the cytokines present are the following: tumor necrosis factor α (TNFα), IL-1, IL-6, IL-8 as well as others.[7,10,11] However, of the cytokines reported to be present in the RA joint, TNFα is believed to be pivotal to the disease process.[1,7] Since TNFα initiates a cascade of pro-inflammatory cytokines,[7,10,11] the central role of this cytokine in the disease process is not surprising. Several investigators have reported that the primary source of TNFα in an RA joint is the Mø;[12,13] however, the factor(s) which are responsible for stimulation of these cells to secrete TNFα have not be identified.

Since it has been reported that neutrophils are abundant in the synovial fluid of the RA joint,[2,3,5] one would also expect myeloperoxidase (MPO) to be present. In fact, other investigators have documented the presence of enzymatically active MPO as well as 16–29 μg/ml of enzymatically inactive MPO in RA joints.[14,15] These studies indicate that both forms of the enzyme may be associated with RA.

In other studies completed in this laboratory, data indicated that recombinant human MPO and enzymatically inactive MPO enhanced the following resident murine Mø functions: (1) respiratory burst,[16,17] (2) secretion of TNFα, IL-6, IL-8, and granulocyte-Mø colony stimulating factor (GM-CSF) (unpublished data), and (3) phagocytosis and intracellular killing of *Candida albicans* and *E. coli*.[16–18] Myeloperoxidase also induced elevated titers of TNFα and IFN α/β *in vivo*.[19] Since it has been reported that neutrophils, MPO, enzymatically inactive MPO, and Mø are present in an inflamed RA joint, the present study was undertaken to determine if the interaction between

either MPO or enzymatically inactive MPO and Mø could contribute to the 'chronicity' of inflammation associated with experimental RA. A Lewis rat model was employed to investigate the effect of neutrophilic MPO on exacerbation of this disease. The disease was induced using a single intra-articular injection of streptococcus A cell wall fragments (PG-APS). After development of experimental RA, either porcine MPO or partially inactivated porcine MPO was injected into the same joint to determine what effect this enzyme had on exacerbation of the disease. For convenience, in the remainder of this manuscript, RA will be used to designate mono-articular experimental RA unless otherwise stated.

Materials and Methods

Myeloperoxidase

The use of rat MPO would have been preferred, but it was not available. Highly purified porcine MPO was generously provided by Dr R.C. Allen. The mannose content of this preparation was 4.6% and it contained 1539 units/ml of activity using o-dianisidine as a substrate. Cathepsin G, a serine protease, has been reported to be present in neutrophils. Trypsin, another serine protease, was used to generate inactive MPO as follows: Myeloperoxidase was partially inactivated by exposure to trypsin-Sepharose 4B gel slurry (Worthington Biochemicals, Freehold, NJ). Prior to mixing the MPO and the gel slurry together, a 1:12 dilution of MPO was heated to 70°C for 10 min. After the solution cooled to below 37°C, the diluted MPO was mixed with the slurry at a 4:1 ratio of MPO to trypsin for the digestion. Digestion was allowed to proceed for 6 h at 37°C. After this incubation, the supernatant was collected, aliquoted, and stored at –20°C until used. This preparation of MPO exhibited 123 units/ml of activity as measured by o-dianisidine. For the remainder of this manuscript, the partially inactivated MPO will be referred to as iMPO.

Animals

Age matched female Lewis rats weighing approximately 180 g were obtained from Harlan Sprague Dawley, Indianapolis, IN. Animals were cared for and housed in a facility according to the guidelines of the Animal Welfare Act.

Reagents

The *Limulus* amebocyte lysate (LAL) assay was used to test all reagents for LPS contamination. The stock MPO preparation contained ≤0.08 ng/ml of LPS. The amount of LPS in the inactivated MPO was the same after exposure to trypsin beads. Materials for the LAL assay were purchased from Associates of Cape Cod (Woodshole, MA). Immunocytochemistry reagents which were purchased from Sigma (St Louis, MO) included: diethylpyrocarbonate, Mayer's hematoxylin solution, bovine serum albumin (fraction V) (BSA), and diaminobenzidine. Other reagents which were purchased from Fisher Scientific (Pittsburgh, PA) were: Superfrost-plus microscope slides, xylene, permount, and TRIS base. *Saccharomyces cervisiae* was the source of mannans which was purchased from Fluka Biochemika, Switzerland. Mannosylated bovine serum albumin (M-BSA) was purchased from E.Y. Laboratories (San Mateo, CA).

Cells

Resident rat peritoneal Mø were collected by peritoneal lavage similar to the procedure previously described by the present investigators.[17,18] Briefly, after the cell suspension was removed from the abdominal cavity and centrifuged at 1000 rpm for 10 min at 4°C, red blood cells were lysed using hypotonic phosphate buffered saline (PBS). After centrifugation, the supernatants were decanted and the peritoneal cells diluted to 1.2×10^6 cells/ml and resuspended in Dulbecco's modified Eagle's medium (Gibco, Long Island, NY) containing HEPES (Sigma), sodium bicarbonate, and gentamicin sulfate (Sigma). Subsequent to a 2-hour incubation, monolayers were washed vigorously with media to remove non-adherent cells. Using staining procedures, it was determined that the adherent cell cultures were ≥99% pure Mø. Subsequently, Mø monolayers were exposed to different concentrations of either MPO or iMPO. After various incubation periods, supernantants were collected and stored at –70°C until used. ELISA kits for TNFα were purchased from Genzyme (Cambridge, MA), and the manufacturer's instructions were followed.

Arthritis Model

Female Lewis rats were used for the induction of a mono-articular arthritis. The right ankle joint (which was the negative control) was injected with 10 µl of normal saline. The left ankle joint (which was the experimental joint) was initially injected with 10 µl of a sterile, aqueous suspension of PG-APS (a generous gift from Dr S. Lichtman, University of North Carolina, Chapel Hill, NC) containing a dose equivalent to 2 µg of rhamnose.[20,21] A 27 gauge 0.5 inch needle was inserted though the Achilles tendon above the calcaneous into the vicinity of the tibiotalar joint. For all injections, the animals were sedated by ether inhalation.

After IA injection of PG-APS, the joints increased in diameter by approximately 1–2 mm (5.8 to 7.0–8.0 mm) within 24–48 h. Over a period of 7–21 days, these joints decreased in swelling to approximately 6.5 mm. Since no further decrease in diameter

was noted over a period of 2 months, 6.5 mm was considered to be 'baseline' level of swelling. Therefore, when a PG-APS treated joint measured 6.5 mm, both ankle joints were re-injected: the right joint received 10 µl of normal saline, and the left joint received 10 µl of various treatment solutions. Those joints which increased in size by a minimum of 0.5 mm 24–48 hours after re-injection were considered positive for inflammation. On a daily basis, the peri-articular swelling of both treated and control ankle joints was monitored. The peri-articular swelling was measured with a caliper starting on day zero, and continued daily until the study was completed.

Histology

Post-mortem, both hind legs were harvested and processed as previously described by other investigators.[20–22] The severity of the arthritis was scored on a scale of 0–3 for synovial hyperplasia and inflammation, tissue erosion, and the presence of neutrophils. All histological sections were read blinded by Suzanne Graham, MD, Department of Pathology at Texas Tech University Health Sciences Center.

Immunocytochemistry

Post-mortem, treated (left) and control (right) ankle joints were harvested and prepared for immunocytochemistry.[22] Sections were placed on Superfrost/Plus slides and deparaffinized. Subsequently, sections were treated with 3% H_2O_2 to destroy endogenous peroxidase activity. After H_2O_2 treatment, sections were incubated with a rabbit polyclonal antibody to TNFα (Calbiochem, La Jolla, CA) overnight at 4°C. Normal rabbit IgG (Sigma) was used as a control. After several washes with PBS buffer, sections were incubated with biotinylated anti-rabbit IgG for 30 min at room temperature. Incubation for an additional 30 min was performed with an avidin:biotinylated enzyme complex (VECTASTAIN ABC system, Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a substrate. Subsequently, sections were counterstained with hematoxylin and examined microscopically.

Statistical analysis

A two-tailed Student's *t*-test was used to determine significance between groups. Most data are presented as the mean ±S.E.M.

Results

Since it is known that TNFα is pivotal to human RA and initiates a cascade of pro-inflammatory cytokines, initial experiments were done to determine if either
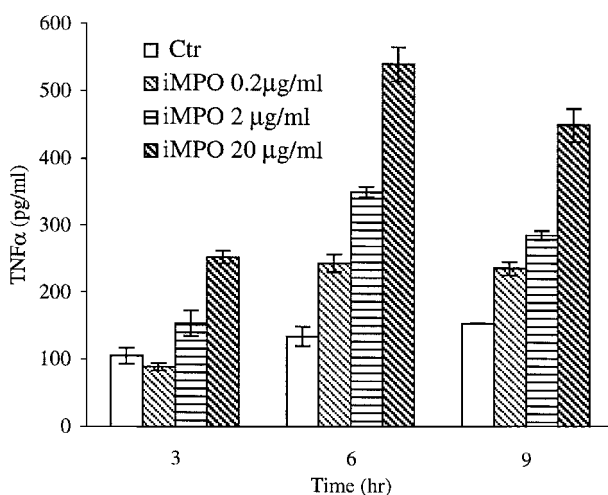

FIG. 1. Induction of tumor necrosis factor-alpha (TNFα) by enzymatically inactive myeloperoxidase (iMPO). Resident rat peritoneal macrophage (Mø) were cultured in a 96 well Costar plate. After a 2-hour incubation, the cells were washed, and either treatments or media alone were added. The supernatants from treated and control wells were collected and stored at –70°C until used for an ELISA. Manufacturer's instructions were followed.

MPO or iMPO could induce TNFα secretion by Mø *in vitro*. Exposure of resident rat peritoneal Mø to either MPO or iMPO induced markedly higher titers of TNFα than that obtained from the control cultures. It can be noted from Fig. 1 that iMPO induced a dose-dependent increase in TNFα secretion with peak titers being obtained at 6 h. If, however, MPO was employed, an increase in TNFα was observed with 20 µg/ml but not with 0.2 µg/ml. At the highest concentration (20 µg), MPO induced approximately 50% less cytokine than iMPO (data not shown). It should be noted that the level of endotoxin in the most concentrated preparation of either MPO or iMPO employed in these studies contained $\leq 8 \times 10^{-3}$ ng/ml. This level of endotoxin did not induce TNFα *in vitro* as determined by ELISA (data not shown).

The next set of experiments was done *in vivo* to determine if either form of the enzyme alone could *initiate* arthritis. After an initial injection of saline, animals were injected weekly for 3 consecutive weeks with various concentrations of either MPO or iMPO. In addition, a non-specific protein, mannosylated BSA, was also tested. None of these substances initiated the clinical symptoms of arthritis as noted by swelling and erythema. Also, since LPS contamination is ubiquitous, and this substance activates Mø, levels of endotoxin (10 ng/joint) that were approximately 1×10^5 times higher than the concentration found in any of the solutions employed in this study were injected IA. The same results as that for other initiators were obtained, i.e. there were no clinical symptoms of arthritis as determined by swelling and erythema (data not shown).
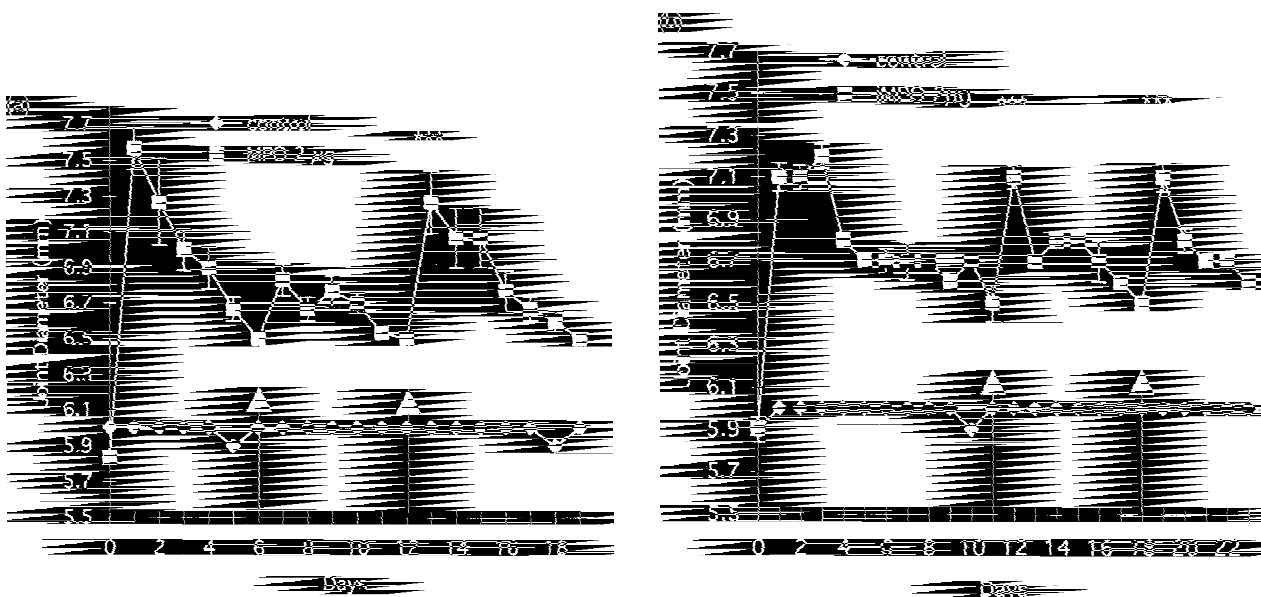
FIG. 2. (a) Exacerbation of peri-articular swelling after re-injection of pre-treated joints with enzymatically active myeloperoxidase (MPO). On day 0, animals were injected with 10 μl PG-APS (2 μg of rhamnose/joint) in the left ankle joint and 10 μl normal saline in the right ankle joint. After the treated joint returned to 'baseline' level of swelling/erythema, the exacerbation regimen was started. On days 6 and 12 (as depicted by the arrows), animals were re-injected with 10 μl MPO (left ankle joint) and 10 μl normal saline (right ankle joint). These values represent the results of at least 3–5 animals. The experiment was repeated at least once. ***$P \leq 0.001$. (b) Periarticular swelling after re-injection of PG-APS pre-treated joints with enzymatically inactive myeloperoxidase (iMPO). On day 0, animals were injected with 10 μl PG-APS (2 μg of rhamnose/joint) in the left ankle joint and 10 μl normal saline in the right ankle joint. After the treated joint returned to 'baseline', the exacerbation regimen was started. On days 11 and 18 (as depicted by the arrows), animals were re-injected with 10 μl iMPO (2 μg/joint): left ankle joint and 10 μl normal saline (right ankle joint). Graphed values represent the results of 3–5 animals. The experiment was repeated at least once. The concentrations reflected in the graph are those present in the joint. ***$P \leq 0.001$.

Since it had been established that LPS at the above concentration could not *initiate* arthritis, a number of animals were injected IA with LPS *after* PG-APS treatment. When 10 ng/joint of LPS were injected IA, there were no clinical symptoms observed. It should be noted that this cycle was repeated twice with identical results. When 15 μg of BSA (a substance to which Lewis rats should not respond)[23] was repeatedly injected IA after PG-APS, once again, no clinical symptoms were observed (data not shown).

Various concentrations of either MPO or iMPO were employed *after* arthritis was initiated with PG-APS. Concentrations employed varied between 0.2 and 20 μg in 10 μl. If 0.2 μg was injected IA, approximately 50% of the animals exhibited RA after each injection series. If 20 μg were employed, all animals developed RA with 66% displaying 'chronic' arthritis. That is, the joints did not return to 'baseline' swelling within a 2 month period after the initial MPO injection (data not shown). If 2 μg of MPO were injected after PG-APS, the initial response was not positive, whereas, a second injection exacerbated a flare of the disease (Fig. 2a). However, when 2 μg of iMPO were injected after PG-APS, all animals exhibited swelling and erythema in the injected joints. This pattern was repeated after a second IA injection of iMPO was administered (Fig. 2b). Because of the

above responses, 2 μg/10 μl were employed for the remainder of the study.

As stated previously, *in vitro* experiments had demonstrated that either MPO or iMPO could induce TNFα. Therefore, experiments were done to determine if TNFα was pivotal in the exacerbation of arthritis observed after IA injections of either MPO or iMPO. After joints were injected with PG-APS and swelling as well as erythema had returned to 'baseline', IA injections of MPO alone, iMPO alone, anti-TNFα alone, MPO and anti-TNFα, or iMPO and anti-TNFα were administered. Figure 3 depicts the results of an MPO+anti-TNFα experiment. The presence of anti-TNF-α completely ablated the effect of MPO with respect to exacerbation of a flare of RA. Similar results were obtained with iMPO (data not shown).

Myeloperoxidase is known to be taken up by Mø via the Mø mannose receptor (MMR).[24] It was hypothesized that blocking the MMR should inhibit clinical symptoms. Mannans are frequently used to block the MMR;[25] therefore, after joints were injected with PG-APS and had returned to 'baseline' level of swelling, IA injections were administered with one of the following: mannans alone, MPO alone, iMPO alone, MPO and mannans, or iMPO and mannans. Mannans alone did not elicit any joint swelling or erythema. When MPO was injected in combination with mannans, the same results were obtained as
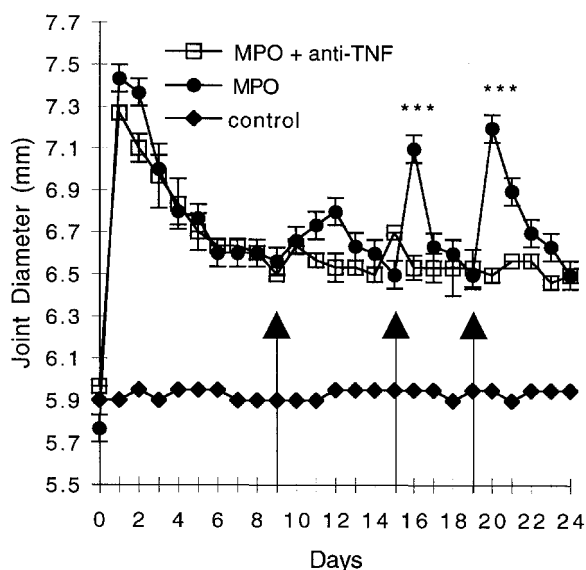
FIG. 3. The inhibition of peri-articular swelling after simultaneously injecting enzymatically active myeloperoxidase (MPO) and anti-tumor necrosis factor-alpha (anti-TNFα). On day 0, animals were injected with 10 µl PG-APS (2 µg of rhamnose/joint) in the left ankle joint and 10 µl normal saline in the right ankle joint. After the treated ankle joint returned to 'baseline' levels of swelling and erythema, the re-injecting regimen was started. On days 9, 15, and 19 (as depicted by the arrows), animals were re-injected simultaneously in the left ankle joint with one of the following: 10 µl MPO alone (2 µg/joint), or a combination of 5 µl MPO (2 µg/joint) and 5 µl anti-TNFα. The right ankle joint was re-injected with 10 µl normal saline. These values represent the results of 3–5 animals. The experiment was repeated at least once. The concentrations reflected in the graph are those injected into the joint. ***$P \leq 0.001$.
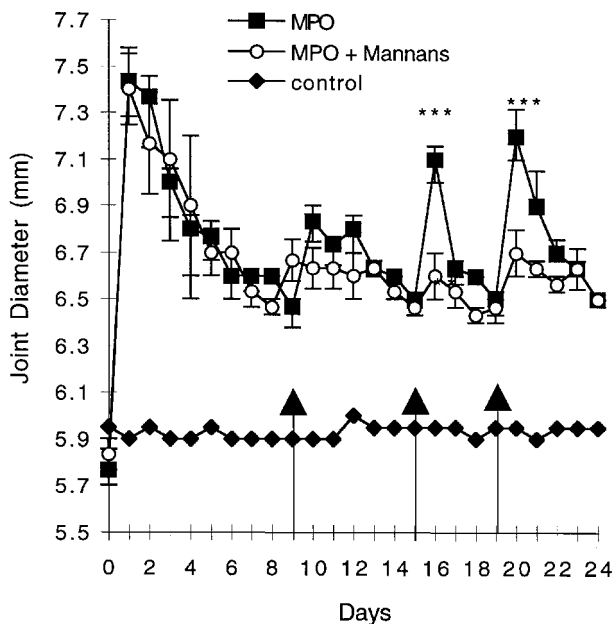

FIG. 4. The inhibition of peri-articular swelling after injecting simultaneously enzymatically active myeloperoxidase (MPO) and mannans. On day 0, animals were injected with 10 µl of PG-APS (2 µg rhamnose/joint) into the left ankle joint and 10 µl normal saline into the right ankle joint. After the treated ankle joint returned to 'baseline', the re-injecting regimen was started. On days 9, 15, and 19 (as depicted by the arrows), animals were re-injected simultaneously in the left ankle joint with one of the following: 10 µl MPO alone (2 µg/joint), or a combination of 5 µl MPO (2 µg/joint) and 5 µl mannans. The right ankle joint was re-injected with 10 µl of normal saline. Graphed values represent the results of 3–5 animals. The experiment was repeated at least once. The concentrations reflected in the graph are those injected into the joint. ***$P \leq 0.001$.

those for mannans alone (Fig. 4), i.e. no effect. Similar results were obtained with iMPO (data not shown).

Histological examinations of joints which received the various treatment modalities were done. Table 1 describes the observations obtained. Minimal to no pathology was noted in joints injected with saline. Minimal pathology did persist at 1 month after a single injection of PG-APS. If MPO was employed as an *initiator* and injected once a week for 3 weeks at the high dose of 20 µg in 10 µl, the pathology

Table 1

Treatment	Synovial hyperplasia	Synovial inflammation	Percentage PMN	Exudate	Bone erosion
Saline[a]	0	0	0	0	0
MPO (20 µg)[a]	+	+	0	0	0
PG-APS[b]	+	+	+	0	0
MPO (20 µg)[c]	+++	+++	+++	+++	+++
iMPO (2 µg)[c]	++	+++	++	+++	++
MPO (2 µg)[c]	+++	+++	+	+	+++
MPO (2 µg) + Mannans (150 µg/ml)[c]	++	++	+	+	++
BSA[c]	+	+	+	+	+

[a] Values represent the pathology observed after 3 weekly IA injections without an initial injection of PG-APS.
[b] Pathology at 3 weeks after a single injection of PG-APS and 3 weekly injections of saline.
[c] Values represent the pathology observed after a single 10 µl injection of PG-APS followed by 3 IA injections of the stated treatment. Each injection was administered after the joint had returned to baseline level of swelling. Amounts in parenthesis represent the concentration injected in the joint. Data represent the mean of at least 4 different animals.
Key: Synovial hyperplasia; 0 = 0–2 layers, + = 3,4 layers, ++ = 5,6 layers, +++ = >6 layers. Synovial inflammation; 0 = <2 lymph, + = inflamm (not crowded), ++ = inflamm (cell crowded), +++ = diffuse. %PMN; 0 = <1%, + = <10%, ++ = 11–20%, +++ = >30%. Exudate; 0 = <1%, + = <10%, ++ = 11–20%, +++ = >30%. Bone erosion; 0 = none, + = only edge 1–2 joint surfaces or juxta bone, ++ = more extensive over surface or 2–5 foci, +++ = more extensive or >5 foci.
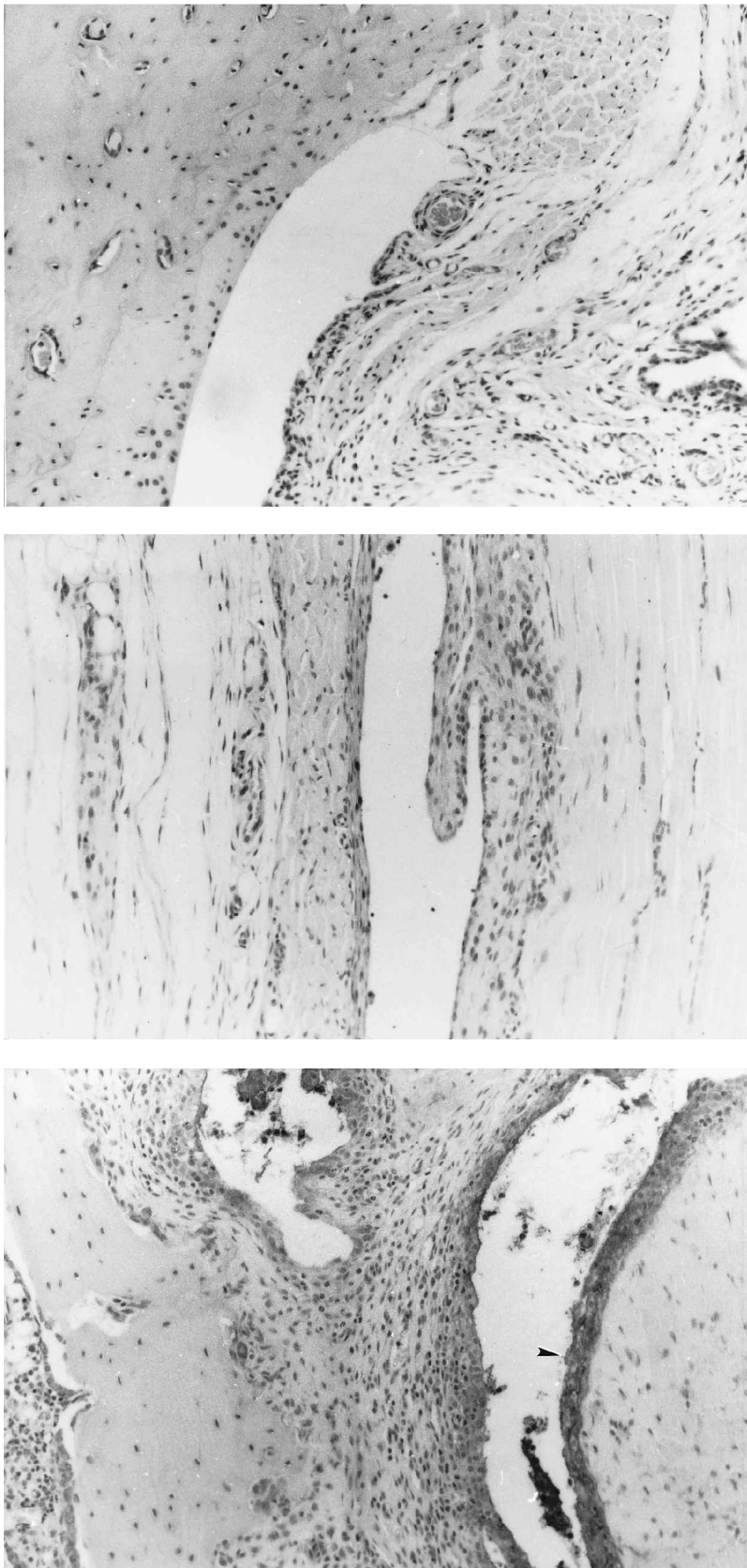
FIG. 5. Photomicrographs showing the detection of tumor necrosis factor-alpha (TNFα) by immunocytochemical (ICC) methods in the synovial tissue of experimental arthritic joints. Tissue was stained with VECTASTAIN ABC system and counterstained with hematoxylin. (a) Photomicrograph of a 'control' ankle joint which was repeatedly injected with saline. For the purpose of ruling out non-specific binding, sequential cuts of 'control' ankle joints were subjected to either anti-TNFα or normal rabbit IgG. (b) Photomicrograph of a joint injected with a single injection of PG-APS and harvested at the same time as the joints pictured in (a) and (c). (c) Distribution of TNFα positive cells in synovial tissue from rat ankle joints that were primed with PG-APS and re-injected with enzymatically active myeloperoxidase (MPO).

observed was similar to that observed with a single injection of PG-APS alone (after 3 weeks). That is: the pathology observed after three weeks was agreeable with what has been observed previously in the model. However, if RA was *initiated* with PG-APS and the joint was subsequently injected with 20 µg of MPO, there was extensive pathology (Table 1). If joints were injected once with PG-APS and then with 2 µg in 10 µl of either MPO or iMPO, the pathology was less severe than that observed with the higher dose of the enzyme. No marked difference in pathology was noted between animals injected with 2 µg of either MPO or iMPO. The presence of mannans diminished the overall pathology observed (Table 1).

Since either MPO or iMPO induced clinical and pathological changes consistent with an *exacerbation* of arthritis, immunocytochemistry studies were done to determine if the Mø-like cells, which comprise the synovial lining of the inflamed joint, were a source of TNFα. After initiation of RA with PG-APS and IA injection of either MPO or iMPO, animals were sacrificed and the joints prepared for staining. Figure 5a shows a section of a joint repeatedly injected with saline. Minimal positive staining was observed in widely spaced areas of the joint. Figure 5b shows a section of a joint injected with a single injection of PG-APS. Figure 5c shows a section of a joint injected with PG-APS, allowed to return to baseline, and then repeatedly injected with MPO. Note that those cells lining the joint stained positive for TNFα. If animals were injected with PG-APS followed by repeated injections of iMPO, the staining pattern was essentially the same for TNFα as that obtained when MPO was injected (data not shown). When normal rabbit IgG was employed instead of rabbit anti-TNFα, no non-specific staining was observed.

Discussion

Numerous cell types including neutrophils, monocytes/Mø, and T cells have been reported to be present in the synovial fluid of human RA joints.[4,6,10,26] Also, numerous cytokines are found in the synovial fluid of inflamed joints.[2,7,11] Of all the cytokines present in an RA joint, it is believed that TNFα is pivotal to the disease since it initiates a cascade of cytokines associated with inflammation.[7,10,11] Tumor necrosis factor α, induces the secretion of IL-1[7,10,11] as well as activates osteoclasts in RA joints.[7,11] Moreover, IL-1 causes loss of proteoglycan from articular cartilage which is one of the hallmarks of human RA.[4] The presence of TNFα and IL-1 induces secretion of IL-8 and GM-CSF, as well as an infiltration of both neutrophils and monocytes into an arthritic joint.[4,27] The cytokines, IL-1, TNFα and GM-CSF cause neutrophil degranulation[4,28] which would provide a continuous supply of both MPO and iMPO to the joint.

Cells of Mø lineage have been reported to be the major source of TNFα in a human RA joint[6,11,29] and the intensity of additional Mø migration into the joint correlates with clinical activity of human RA.[6] When resident rat peritoneal Mø were exposed to either iMPO (Fig. 1) or MPO an increase in TNFα secretion was observed. Tumor necrosis factor induction by iMPO was dose dependent; however, this was not true when MPO was employed. This could be explained in part by the following: MPO induced greater amounts of reactive oxygen intermediates (ROI) than MPO (unpublished data), and that ROI can enhance secretion of a cytokine as well as oxidize receptors[30] preventing cell signaling causing a reduction of secreted cytokine. Only at the highest concentration of MPO, were the Mø stimulated sufficiently to secrete TNF. Thus, minimal oxidation by iMPO would result in greater cell signaling and consequently higher titers of TNF (approximately 2-fold). These *in vitro* studies suggest that both MPO and iMPO can interact with Mø in the microenvironment of an arthritic joint.

It has been established that PMN's within human RA synovial fluid degranulate and secrete MPO into the microenvironment.[5] Within 10 minutes of stimulation of neutrophils, approximately 30% of their MPO is secreted.[31] Of the 30% of the secreted enzyme, only 5% retains enzymatic activity.[31,32] Results of other studies indicate that PMN's released approximately 50% of their MPO at a site of inflammation. Also, with respect to human RA, Davies *et al.*, reported the presence of MPO in arthritic joints.[5] Moreover, Edwards *et al.*, have reported that there are 16–29 µg/ml of enzymatically inactive MPO in the synovial fluid of a human arthritic joint.[15] In the present study, the injection of 2 µg/joint (200 µg/ml) of the enzyme was the standard concentration employed. Since there is not a consensus among the numerous studies completed to date with respect to the amount of *both* MPO and iMPO in a human RA joint, concentrations utilized in this study may well be within 'pathological range'.

From 0.2–20 µg of MPO injected into a joint did not *initiate* RA within 24–48 h after injection, as determined by both clinical and pathological criteria. In this study, IA injections of either MPO or iMPO *after* PG-APS induced arthritis. The fact that PG-APS, but not MPO or iMPO, could initiate arthritis could be explained in part by the fact that PG-APS is a more potent inducer of TNFα. Also, it is possible that MPO, which is highly conserved in various species, is not highly immunogenic. With respect to prolonged presence of this enzyme and the induction of RA, it has been reported that highly cationic molecules have been associated with retention in joints and arthritogenicity.[23] Retention is believed to be due to the interaction of the cationic molecule with the negatively charged cartilage.[23,34,35]

Since this study involved repeated injections of various treatments into a particular joint, there was concern that the trauma alone of repeated injections could induce inflammation associated with RA. Therefore, BSA and/or saline was repeatedly injected into PG-APS treated joints. The results indicated that repeated injections were not sufficient to induce marked clinical or pathological changes (Table 1).

Exacerbation of an arthritic flare can be induced by a number of exogenous substances such as LPS (at higher doses than those employed in the present study) and microbial superantigens.[33] The purpose of 'cycling' the injections of either MPO or iMPO after an initial injection of PG-APS was to simulate flares of the disease which occurs in patients (Figs 2a and b). As stated previously, the first injection of MPO, after PG-APS, did not induce a positive flare whereas the first injection of iMPO did induce an exacerbation of the disease. A possible explanation for this could be the fact that MPO induces tissue damage through ROI.[30] Therefore, the first injection would result in predominantly tissue damage and subsequent immune cell recruitment. However, with the second injection, immune cells would now be present in the joint and would induce higher titers of TNFα. With iMPO, an initial injection would favor induction of proinflammatory cytokines and rapid recruitment of immune cells and not direct tissue damage due to ROI.

Shepherd and Hoidal reported that MPO binds to Mø via a specific receptor, the MMR.[24] Therefore, if either MPO or iMPO was causing an exacerbation of experimental arthritis and contributing to 'chronicity' of the disease, then inhibiting the binding of this enzyme should ameliorate clinical symptoms. When either MPO or iMPO were injected simultaneously with mannans, there was no exacerbation of clinical disease (Fig. 4) providing evidence for the importance of Mø-MMR-neutrophil (i.e. MPO or iMPO) interaction. Pathology reports corroborated these results (Table 1). Since PG-APS is a polymer of acetylated mannose, the same concept was applied to the model itself. If MMR-PG-APS interaction was sufficient to induce RA, then via competitive binding, mannans should diminish or prevent the initiation of the disease. When PG-APS was injected simultaneously with mannans, there was no clinical response observed. Again this indicates a central role of the Mø-MMR ligand interaction in the induction of arthritis using this animal.

Synovial type A cells, which are of Mø lineage, are a major source of TNFα in arthritic joints.[8] Using immunocytochemistry, TNFα was found only among the mononuclear phagocytic cells of the lining of the joint, in perivascular areas, and in the pannus[22] of joints injected with PG-APS followed by injection of either MPO or iMPO. These results indicate that either MPO or iMPO interacting with cells of the synovial lining, which are of Mø-lineage, induce the production of TNFα. Also, the pattern of TNFα staining parallels that observed in human RA.

Various investigators have reported that T cells participate in the pathological events of RA.[36,37] However, recognition of the neutrophil as a major contributor to the pathology associated with RA has only recently been reported.[26] The results of the present study have described the importance of the neutrophil-Mø interaction in exacerbation of inflammation associated with a model of arthritis. These results indicate that: (1) either MPO or iMPO induce the secretion of TNFα by rat Mø *in vitro* or *in vivo*; (2) neither form of the enzyme can *initiate* RA in the rat model; (3) numerous exacerbations of arthritis are initiated by repeatedly injecting either MPO or iMPO into a joint pre-treated with PG-APS; (4) immunocytochemistry studies indicated the presence of TNFα; (5) the presence of anti-TNFα inhibits either MPO or iMPO induced exacerbation of PG-APS-induced arthritis, and (6) clinical and pathological symptoms of arthritis can be ameliorated or ablated by blocking the MMR which is one of the major receptors involved in the uptake of this peroxidase by the Mø.

In conclusion, there appears to be a previously unrecognized interaction of either MPO or iMPO with Mø which could partially explain the 'chronicity' of inflammation observed in this model. A recent report indicated that 'the products of the MPO-H_2O_2-Cl pathway can act as immunological modifiers providing a further pathway linking acute and chronic inflammation and innate and adaptive immune responses'.[9] Further studies are needed in order to answer the question as to whether this interaction plays a role in 'chronic' inflammation associated with human RA.

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