ENDOTHELIAL ACTIVATION IN MONOSODIUM URATE MONOHYDRATE CRYSTAL-INDUCED INFLAMMATION

In Vitro and In Vivo Studies on the Roles of Tumor Necrosis Factor α and Interleukin-1

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Objective. There is relatively little direct evidence for the roles of interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) in activating endothelium in vivo. The aim of this study was to use in vitro and in vivo models to investigate the contribution of these cytokines to both E-selectin expression and the recruitment of polymorphonuclear cells (PMN) in monosodium urate monohydrate (MSU) crystal-induced inflammation.

Methods. MSU crystals were incubated with freshly isolated mononuclear cells, after which the harvested supernatants were tested for their ability to induce E-selectin expression during coculture with human umbilical vein endothelial cells. Subsequent experiments were performed with the addition of neutralizing anticytokine antibodies/antisera. The role of TNFα was then studied in an MSU crystal-induced monarthritis model, in the presence or absence of anti-TNFα (5 mg/kg intravenously). 99mTc-(technetium) and 111In-(indium)-labeled PMN cells and 1.2B6 anti-E-selectin monoclonal antibody (MAb) were intravenously administered 4 hours after intraarticular injection to quantify PMN recruitment and E-selectin expression in inflamed joints.

Results. MSU crystals were a potent stimulus for IL-1 and TNFα production by monocytes in vitro, and these cytokines fully accounted for MSU crystal-stimulated, monocyte-mediated endothelial activation. In the MSU crystal-induced monarthritis model, TNFα blockade was very effective in suppressing both E-selectin expression and PMN emigration into the inflamed joints, as judged by gamma-camera image analysis and postmortem tissue counting following the intravenous injection of 99mTc-PMN and 111In-anti-E-selectin MAb.

Conclusion. IL-1 and TNFα appear to be the only factors released by monocytes following incubation with MSU crystals, which induce E-selectin expression in vitro. Anti-TNFα is effective in suppressing endothelial activation and PMN recruitment in vivo. E-selectin imaging can be used to assess the endothelial response to therapy and may prove useful for clinical studies.

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expressed by activated, but not unstimulated, EC (3). Immunohistologic studies have revealed its expression in a wide variety of acute and chronic inflammatory settings (4). In view of these properties, E-selectin provides a useful target for the in situ detection of cytokine-induced endothelial activation. We have recently developed a technique for quantifying and localizing E-selectin expression in vivo in several porcine models of inflammation, in which we use the intravenous administration of a radiolabeled anti-E-selectin monoclonal antibody (MAb), 1.2B6. By implementing this technique, we have demonstrated specific binding of MAb 1.2B6 to activated endothelium in models of systemic vascular activation (5,6), cutaneous inflammation (5,7-9), and arthritis (6,10,11), with the latter studies allowing the imaging of anti-E-selectin MAb accumulation with a gamma camera. This approach has also enabled us to measure endothelial E-selectin expression in vivo in relation to other parameters of inflammation, such as accumulation of neutrophils and lymphocytes (7-9) and the exudation of albumin (8).

Although gouty inflammation is one of the best understood of the inflammatory arthritides, there remain several fundamental questions in relation to its pathogenesis and resolution (12). In particular, while mononuclear phagocytes produce a number of proinflammatory mediators following contact with monosodium urate monohydrate (MSU) crystals, the exact mediators of endothelial activation and leukocyte recruitment in acute gout remain unknown. Although it seems probable that IL-1α/β and TNFα play important roles in these processes (13,14), there is no direct evidence regarding their relative importance, nor is there evidence for their involvement in MSU crystal-induced arthritis in vivo.

In this investigation, we have explored the capacity of MSU crystal-activated mononuclear cells (MNC) to induce EC activation in vitro, by using a panel of neutralizing antibodies to IL-1α/β and TNFα/β. Based upon our in vitro results, we have extended the study to an in vivo investigation of the importance of TNFα for MSU crystal-induced arthritis in the pig, using differentially radiolabeled anti-E-selectin MAb and leukocytes to measure E-selectin expression and leukocyte recruitment in the inflamed joint.

MATERIALS AND METHODS

Antibodies. MAb 1.2B6 is a mouse IgG1 MAb that reacts with both human and pig E-selectin (15,16). Biotinylated MAb 1.2B6 was performed as previously described (17). F(ab')2 fragments of 1.2B6 and of MOPC 21 control IgG1 were prepared and radiolabeled with 111indium (111In) and 125I for in vivo localization studies, as previously described (6). Murine monoclonal anti-ICAM-1 (8.4A6) (18) and anti-VCAM-1 (1.4C3) (15) MAb were used in an enzyme-linked immunosorbent assay (ELISA) for the initial experiments. Neutralizing MAb anti-TNFα (357) and anti-TNFβ (3598111) were generously donated by Dr. J. Saklavata (The Babraham Institute, Cambridge, UK). The anti-porcine TNF MAb (BAY X1351) was produced at Miles Inc. (now Bayer Corp., Berkeley, CA) by hybridoma culture and purified from culture harvests by cell separation, polyethylene glycol precipitation, anion exchange, and size-exclusion chromatography. The final preparation was >99% pure and showed full functional binding to recombinant human TNFα (rHuTNFα) (19).

Cytokines. As a positive control, rHuTNFα (10 ng/ml; the kind gift of Dr. M. Robinson, Celltech Ltd., Slough, UK) was used for endothelial activation in all experiments. TNFβ was purchased from R & D Systems Europe (Abingdon, UK). IL-1α and IL-1β were generously donated by Dr. Jean-Jacques Mermod (Glaxo Institute for Molecular Biology). MSU crystals. The MSU crystals used in this study were from the same batch as used previously (11). Endotoxin levels, as assessed by the limulus lysate assay (chromogenic substrate method) (20), were <5 pg/mg (<0.0625 endotoxin IU/mg) of suspended crystals.

Isolation and culture of EC. Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords by a modification of the method of Jaffe et al (21) and cultured as previously described (15). Porcine aortic EC were isolated and cultured from aortas of 3-month-old pigs using a similar protocol (16). EC were plated at 3-4 × 10^4 cells per well in gelatin-coated 96-well microtiter plates (Costar, Cambridge, MA) in order to achieve confluent monolayers. Stimulation of EC was performed by adding the appropriate volume of cytokine or MNC supernatant. For experiments involving inhibitory anticytokine MAb, the cytokines or MNC supernatants were first incubated with the inhibitory MAb for 15–30 minutes prior to addition to individual wells containing EC. Following stimulation, EC monolayers were fixed with an aqueous solution of 2% paraformaldehyde (BDH Laboratory Supplies, Poole, UK), 100 mM m-Jysine monohydrochloride (Sigma, St. Louis, MO), and 2.1 mg/ml sodium metaperiodate (Sigma) prior to measurement of antigen expression (22).

Isolation of leukocyte subsets for in vitro studies. MNC were isolated from heparinized peripheral blood by density gradient centrifugation on Ficoll-Hypaque (pyrogen-tested Lymphoprep; Nycomed, Oslo, Norway). MNC banded at the plasma–Ficoll interface were separated, washed 3 times, and resuspended in RPMI 1640 with penicillin and streptomycin (Gibco BRL, Paisley, Scotland) and 5% fetal calf serum (HyClone, Logan, UT). Morphologic analysis of cytocentrifuged samples of the MNC preparations revealed a composition of ~83% lymphocytes, 12% monocytes, <3% neutrophils, and only occasional red blood cells and platelets. Monocytes...
were isolated from single-donor, platelet-depleted buffy coat packs acquired from the Blood Transfusion Service (Colindale, North London, UK) by elutriation in a Beckman J6-MC elutriator (Beckman Instruments, Palo Alto, CA) as previously described (23).

T lymphocytes were obtained from the washed MNC preparations. Briefly, MNC were panned on tissue culture dishes precoated with purified anti-HLA-DR MAb L243 (American Type Culture Collection, Rockville, Maryland) and goat anti-human immunoglobulin for 60 minutes at 37°C. Nonadherent cells were eluted and incubated with a cocktail of purified MAb L243, mouse anti-human Ig (Fab-specific), and Leu-19 (anti-CD56; Becton Dickinson, Twickenham, UK). Excess antibodies were removed by washing, and the cells were depleted of monocytes, B cells, and natural killer cells by 2 rounds of magnetic immunodepletion using sheep anti-mouse—coated magnetic beads (Dynal, Oslo, Norway). Purity of monocyte and T cell preparations was >93% and >98%, respectively, as judged by flow cytometric staining of CD14 and CD3.

Isolation and radiolabeling of leukocytes for in vivo studies. Porcine polymorphonuclear leukocytes (PMN) were isolated from peripheral blood that was obtained following the insertion of a central venous catheter. The PMN were labeled with 99mTc-hexamethylpropyleneamine oxine (Ceretec; Amersham International, Amersham, UK) as previously described (6). The composition of the preparation was assessed by morphologic analysis of cytocentrifuged samples, and was found to contain 95% neutrophils and 2-3% eosinophils, with the remainder made up of occasional monocytes and lymphocytes.

Detection of surface antigens on cell monolayers. The expression of cell surface antigens on fixed EC monolayers was measured by cell-based ELISA, as previously described (15), using biotinylated rabbit anti-mouse Ig F(ab')2 fragments for detection of binding of the primary antibody. Biotinylated anti-E-selectin MAB 1.2B6 was used as the primary MAB in experiments including anticytokine antibodies. Following washing, binding of biotinylated antibody was detected by incubation with a high molecular weight complex of streptavidin—biotin—horseradish peroxidase (Dako, Carpinteria, CA). The integrity of the monolayer was then inspected under phase-contrast microscopy. Assays were developed, by the addition of 200 μl per well of 0.5 mg/ml α-phenylenediamine (Sigma), 0.03% hydrogen peroxide (volume/volume) in a citrate-phosphate buffer (pH 5.0 substrate, 200 μl/well). In assays using MSU crystal suspensions, protein estimation using crystal violet was performed to exclude differential cell loss between cultures. The wells of the microtiter plates were washed with phosphate buffered saline, and 100 μl of crystal violet (Sigma; 0.1% weight/volume in distilled water) was added. The stain was carefully washed away after 10 minutes and 100 μl of 33% (v/v) acetic acid was added. The optical density was measured at 620 nm.

Experimental protocol for in vitro studies. In the initial experiments, 200 μl of freshly isolated MNC (10⁶ cells/ml) was plated into individual wells of 96-well U-bottomed microtiter plates (Nunclon, Roskilde, Denmark). MNC were then incubated in the presence or absence of MSU crystals, at a final concentration of 0.5 mg crystals/ml, for varying durations between 1 hour and 24 hours at 37°C. Following centrifugation (200g), 100 μl of MNC supernatant was harvested from each well, pooled into 200-μl aliquots, and stored in sterile vials at −70°C. The pooled MNC supernatants were then added (50 μl/well) to freshly plated HUVEC and incubated for 6 hours, 10 hours, or 24 hours for measurement of E-selectin, VCAM-1, and ICAM-1 expression, respectively. TNFα and IL-1α (final concentration 10 ng/ml each) were used as positive controls, and unstimulated MNC supernatants and HUVEC medium alone served as negative controls.

Adhesion molecule expression was subsequently determined by ELISA. Stored MNC supernatant aliquots from each experiment were reserved for the later quantification of levels of immunoreactive cytokines. These experiments were repeated using purified monocytes and T lymphocytes (200 μl/well, 10⁶ cells/ml). In several experiments, serial dilutions of MSU crystals (0.5 mg/ml) were coincubated with freshly plated HUVEC for 4-6 hours to ascertain any direct effects of the crystals on endothelial activation.

Further experiments were performed using a similar protocol, but included the coinoculation of MNC supernatants with neutralizing mouse anticytokine MAB (anti-TNFα, anti-TNFβ) or polyclonal antisera (anti-IL-1α, anti-IL-1β), either alone or in combination. The inhibitory antibodies were added either before or after incubation of the MNC supernatant with MSU crystals. In each experiment, the neutralizing capacity of each individual antibody was confirmed in control wells by incubation with its corresponding cytokine.

Experimental protocol for in vivo studies. Young Large White pigs, weighing 19-24 kg, were obtained from a commercial supplier (Froxfield Farms, Petersfield, UK) and housed under standard husbandry conditions. Animals were studied according to a protocol approved under the United Kingdom Animals (Scientific Procedures) Act of 1986. Anesthesia was induced for intraarticular and intravenous injections, and for subsequent imaging studies, by the inhalation of halothane, which resulted in rapid induction of sedation with minimal stress. Anesthesia was maintained by repeated intravenous boluses of propofol (1 mg/kg Diprivan; Zeneca Limited, Macclesfield, UK), given every 15-20 minutes.

We have previously reported the anti-E-selectin localization data for the 3 control pigs with MSU crystal-induced arthritis, in a study that validated the feasibility of the imaging approach (11). The present study involved 5 additional animals (Large White pigs). In order to establish that the anti-TNFα antibody preparation could neutralize TNFα activity in vivo, we initially studied its inhibitory effect on localized cutaneous inflammation induced by the injection of TNFα, using sites injected with IL-1α as control lesions. For this experiment, 2 pigs were studied, 1 of which received 5 mg/kg of anti-TNFα intravenously 2 hours prior to the induction of the inflammatory lesions, and the other of which served as a control. Inflammatory skin lesions were induced by IL-1α (7,500 units) and TNFα (2,250 units, 4,500 units, and 9,000 units) 2 hours prior to the end of the experiment (5,7). Ten minutes prior to termination of the experiment, each animal received, intravenously, 111In(indium (111In)—labeled anti-E-selectin F(ab')2 MAB (200 μg, 2.7 ± 0.9 MBq) (mean ± SD), 99mTc-labeled neutrophils (1.34 ± 0.09 × 10⁶ cells, 21.9 ± 0.5 MBq), and 125I-labeled MOPC 21 (control Ig, 250 μg, 1.2 ± 0.3 MBq). The skin lesions were subsequently excised and counted.

For the arthritis model, 3 animals were studied using
Arthritis was induced in the right (inflamed) knee by the intraarticular injection of 20 mg of MSU crystals suspended in 1 ml of sterile saline. The left (control) knee was injected with 1 ml of saline alone. The cytokines IL-1α (7,500 units) and TNFα (4,500 units) were also injected intradermally at this time to act as negative and positive controls, respectively, for the effects of anti-TNFα. Four hours after the intraarticular injection, 111In-labeled 1.2B6 F(ab')2 fragments (200 µg, 16.5 ± 1.1 MBq), 99mTc-PMN (1.75 ± 0.32 × 10⁶ cells, 192 ± 10.2 MBq), and 125I-labeled MOPC 21 (250 µg, 1.3 ± 0.2 MBq) were administered intravenously. The animals were then imaged at 7 hours and 24 hours post–intraarticular injection.

**Imaging.** Dual-isotope (111In and 99mTc) static imaging of the knees was performed using a gamma camera (Maxi Camera, General Electric, Horsholm Denmark) with the pigs in a supine position at 7 hours and 24 hours post–intraarticular injection (i.e., 3 hours and 20 hours post–intravenous administration of the radioisotopes). To quantify uptake of the tracers on scintiscans, regions of interest (ROI) were drawn over both joint spaces, and the increase in image intensities (expressed as an image localization increment [ILI]) between inflamed and noninflamed knees was calculated for 111In and 99mTc, following correction for ROI size, radioisotope background, and crossover activity. The ILI in the inflamed knee was calculated as follows:

\[
ILI = \frac{\text{pixel counts, inflamed knee} - \text{pixel counts, noninflamed knee}}{\text{pixel counts, noninflamed knee}}
\]

125I is not suitable for imaging. Therefore, scintigraphic data were not available for the control Ig MOPC 21.

**Radioactivity counting.** Following completion of the imaging studies, the animals were killed by means of exsanguination under deep anesthesia, and tissue samples were excised for subsequent counting. The accumulation of radioactivities in synovia from both inflamed and noninflamed knees of each animal was expressed as a percentage of the injected dose per gram (% ID/gm) of synovial tissue. Increased tissue uptake of radiolabeled tracers in synovial samples from inflamed knees was expressed as a tissue localization increment (TLI), calculated as follows:

\[
\text{TLI} = \frac{(\text{counts per minute/gm tissue, inflamed knee} - \text{counts per minute/gm tissue, noninflamed knee})}{\text{cpm/gm, noninflamed knee}}
\]

**Statistical analysis.** Statistical data were compared using Student's paired t-test.

### RESULTS

**In vitro studies.** Stimulation of MNC by MSU crystals to release factors that induce HUVEC adhesion molecule expression. In an initial set of experiments, we tested the capacity of MSU crystal–activated MNC to stimulate cultured HUVEC. Following incubation with increasing concentrations of MSU crystals (0.1 mg/ml, 0.5 mg/ml, and 1.0 mg/ml) for 4 hours and 24 hours, MNC supernatants were harvested and cocultured with HUVEC. The induced luminal expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 was then measured by ELISA. Supernatants from MNC alone (i.e., in the absence of crystals) demonstrated minimal capacity to up-regulate adhesion molecule expression. In contrast, supernatants from MSU crystal–activated MNC produced a marked up-regulation of adhesion molecule expression, with 0.1 mg/ml of crystals exhibiting a potency similar to that induced by the higher concentrations of MSU crystals (Figure 1). In view of the equivalent up-regulation of the 3 adhesion molecules, E-selectin expression was chosen as the marker of EC activation in subsequent experiments.

To further characterize the capacity of MSU crystal–activated MNC supernatants to stimulate EC, we examined doubling dilutions of the supernatant that was obtained from MNC stimulated with 0.5 mg/ml of MSU crystals. This produced a uniform dose-dependent response, with detectable activity at 1:64 and maximal activity at 1:2 (Figure 2A). Evaluation of the time course of the response showed that MNC released detectable EC activity within 1 hour of incubation with MSU crystals. Near maximal levels of EC activity were achieved by 90 minutes, and were maintained at 24 hours (Figure 2B). The E-selectin–stimulating activity of MNC supernatants appeared to be derived from monocytes, as shown in a separate experiment in which E-selectin–stimulating activity was detected in supernatants from cultures of MSU crystals with isolated monocytes, but not in supernatants of cultures of MSU crystals with purified T lymphocytes (data not shown). We performed several experiments in which we coincubated MSU crystals directly with cultured EC. MSU crystals were toxic to the EC at 0.5 mg/ml and 0.25 mg/ml, and no induction of E-selectin expression was detected following coincubation with lower concentrations (data not shown).

**Role of IL-1 and TNF in the E-selectin–inducing activity in MSU crystal–activated MNC supernatants.** We next performed experiments with neutralizing antibodies to IL-1 and TNFα to determine to what degree IL-1 and TNFα bioactivity accounted for E-selectin expression in response to incubation with crystal-mediated MNC supernatants. The MAb were added to the MNC cultures before addition of MSU crystals (“preincubation”) or were mixed with supernatants following 24-hour crystal/MNC cocultures (“postincubation”). We found that blockade by individual anti-TNF or anti–IL-1 MAb alone was insufficient to achieve any significant reduc-
ADHESION MOLECULES IN URATE CRYSTAL INFLAMMATION

Figure 1. Release by monosodium urate monohydrate (MSU) crystal-activated mononuclear cells (MNC) of mediators that induce adhesion molecule expression on cultured endothelial cells (EC). MNC were cocultured with MSU crystals at concentrations between 0.1 mg/ml and 1.0 mg/ml for 4 hours (■) or 24 hours (□), after which supernatants were harvested and later cocultured with human umbilical vein EC (HUVEC). Optical density (OD) was measured to determine up-regulation of E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) expression by HUVEC, compared with that induced by tumor necrosis factor α (TNF) (10 ng/ml). Results shown are representative of 2 experiments. Bars show the mean and SD of quadruplicates.

Figure 2. Characterization of the capacity of MSU crystal-activated MNC supernatants to stimulate EC. A, After 24-hour incubations of MNC with 0.5 mg/ml of MSU crystals, doubling dilutions of MNC supernatants were tested for their ability to induce E-selectin expression by HUVEC. B, The time course of the response was determined following incubation of MNC with 0.5 mg/ml MSU crystals. Results shown are representative of 2 experiments. Values are the mean and SD of quadruplicates. IL-1 = interleukin-1. See Figure 1 for other definitions.

Figure 3. Characterization of the capacity of MSU crystal-activated MNC supernatants to stimulate EC. A, After 24-hour incubations of MNC with 0.5 mg/ml of MSU crystals, doubling dilutions of MNC supernatants were tested for their ability to induce E-selectin expression by HUVEC. B, The time course of the response was determined following incubation of MNC with 0.5 mg/ml MSU crystals. Results shown are representative of 2 experiments. Values are the mean and SD of quadruplicates. IL-1 = interleukin-1. See Figure 1 for other definitions.

These experiments were performed in a manner identical to that used for human cells, using porcine MNC, porcine aortic EC, and neutralizing antibodies against porcine cytokines. The results were similar to
those with human cells in that a combination of anti-
TNFα, anti-IL-1α, and anti-IL-1β MAb fully inhibited
EC activation, whereas no inhibition was seen following
the use of individual neutralizing MAb (Figure 3B).

In view of the failure to inhibit the E-selectin-
stimulating activity of supernatants from 24-hour cul-
tures with antibodies to single cytokines, we performed
an experiment testing supernatants harvested from hu-
man MNC after only 1 hour and 4 hours of incubation
with MSU crystals. As shown in Figure 4, anti-
IL-1β fully inhibited E-selectin–stimulating activity in
supernatants incubated with crystals for only 1 hour, and
was also significantly inhibitory in 4-hour supernatants.
As seen previously, anti-IL-1β alone had no inhibitory
effect on the capacity of 24-hour supernatants to stim-
ulate E-selectin. Likewise, antibodies against other cy-
tokines had no significant inhibitory effects on superna-
tants from cultures incubated with crystals for 1 hour, 4
hours, or 24 hours. These data strongly suggest that
IL-1β is the dominant E-selectin–stimulating cytokine at
early time points, but that, subsequently, sufficient TNF
is produced to render inhibition with IL-1 alone
ineffective.

In vivo studies. Having established that TNFα
release contributes to the capacity of MSU crystal–
activated monocytes to stimulate E-selectin expression
in vitro, we proceeded to examine the contribution of
TNFα in an in vivo model of MSU crystal–induced
arthritis, using a neutralizing anti-TNFα MAb. In order
to first establish that the preparation of anti-TNFα MAb
was capable of specifically inhibiting TNFα activity in
vivo in the pig, we determined its effect on cutaneous
inflammation induced by rHuTNFα, using IL-1α as a
control inflammatory stimulus. Compared with the find-

Figure 3. Role of TNF and interleukin-1 (IL-1) in the E-selectin–inducing activity of MSU crystal–activated MNC supernatants. A, HUVEC were
incubated with supernatants (SN) from MSU crystal–stimulated MNC in the presence of neutralizing monoclonal antibodies (TNFα and TNFβ)
and/or neutralizing polyclonal antisera (IL-1α and IL-1β), either alone or in combination. The anticytokine antibodies were added to the MNC
cultures either prior to (■) or following (□) incubation with MSU crystals for 24 hours. A combination of antibodies to all 4 cytokines fully
abrogated the endothelial response (\( * = P < 0.01 \)). B, A further set of experiments was performed using porcine MNC, porcine aortic EC, and
neutralizing antibodies to porcine cytokines, with a protocol identical to that used for the human cells. The response was fully inhibited when a
combination of all 3 antibodies was used (\( * = P < 0.01 \)). Results shown are representative of 3 experiments. Bars show the mean and SD of
quadruplicates. See Figure 1 for other definitions.
Figure 4. Dominance of interleukin-1β (IL-1β) in the stimulation of
E-selectin in MNC supernatants (SN) at early time points after
exposure to monosodium urate monohydrate (MSU) crystals. Human
MNC were incubated with MSU crystals for 1 hour, 4 hours, or 24
hours, after which they were harvested and tested, in the presence of
anticytokine antibodies, for their capacity to induce E-selectin expres-
sion by HUVEC. Results shown are representative
of 3 experiments.
Bars show the mean and SD
of quadruplicates. * = P < 0.05 versus
other cultures. See Figure 1 for other definitions.

ings in an untreated animal, anti-TNFα led to marked
inhibition of accumulation of both ⁹⁹ᵐTc-labeled PMN
and ¹¹¹In-labeled MAb 1.2B6 (anti-E-selectin) in the
TNFα-induced lesions (P < 0.01; data not shown), but
had no significant inhibitory effect on uptake of the
tracers into the IL-1α-induced lesions. Cutaneous injec-
tions of TNFα and IL-1 were subsequently used as
positive and negative controls, respectively, for the
effects of anti-TNFα MAb in the experiments described
below.

Injection of MSU crystals (20 mg) into the right
knee of untreated control animals led to obvious joint
swelling, with associated redness and warmth. Signs were
most marked at 4–6 hours postinjection. Scintigraphic
images of ¹¹¹In-1.2B6 uptake in the 3 control animals
both 7 hours and 24 hours after MSU crystal injection
exhibited a marked increase in image intensity in the
inflamed knees, particularly over the joint space, when
compared with the contralateral control knees (11)
(Figure 5A). In contrast, the images obtained over the
inflamed knees of anti-TNFα-treated animals were rela-
tively modest (Figure 5B), in accordance with an ab-
sence of clinically detectable inflammation. The increase
in image intensity of the inflamed knees could be
objectively quantified by determining the ILI, or differ-
eence in uptake between the inflamed knees and the
control knees in the ROI (synovial space) for each
animal. The ILI for ¹¹¹In-1.2B6 was 0.4 ± 0.07 and 0.5 ±
0.1 (mean ± SD) at 7 hours and 24 hours, respectively,
post–intraarticular injection of MSU crystals in the
anti-TNFα-treated animals, which was significantly less
(P < 0.05 for each time point) than the respective values
for the control animals (2.46 ± 0.77 and 2.52 ± 0.76).

Images of synovial uptake of ⁹⁹ᵐTc-PMN in the
inflamed knees of the 3 control animals showed an
increase in image intensity compared with the nonin-
famed knees (mean ± SD ILI 0.97 ± 0.11 and 0.88 ±
0.12 at 7 hours and 24 hours, respectively, post–intraarticular injection), although the differences were
less than with ¹¹¹In-1.2B6. Although there was a reduc-
tion in image intensity in the anti-TNFα-treated animals
(ILI 0.49 ± 0.11 and 0.36 ± 0.18 at 7 hours and 24 hours,
respectively, post–intraarticular injection), the differ-
eence in the ILI between the 2 groups of pigs did not
achieve statistical significance.

Postmortem examination of untreated (control)
animals (24 hours after MSU crystal injection) revealed
the presence of small synovial effusions and macroscopi-
cally inflamed synovium in MSU crystal–injected knees.
In contrast, there were no external signs of synovitis and
no postmortem evidence of joint effusions or macro-
scopic inflammation in the 3 anti-TNFα-treated ani-
mals. Radioactivity of tissue samples taken from the
synovia of MSU crystal–injected knees and control
knees was counted postmortem, and expressed as the %
ID/gm of tissue and as a TLI, the latter reflecting the
degree of increased uptake in inflamed knees. The
individual values for each animal in the control and
anti-TNFα groups are presented in Table 1.

In the untreated animals, there was a marked
accumulation of ¹¹¹In-labeled MAb 1.2B6 (mean ± SD
TLI 20.3 ± 2.3) and ⁹⁹ᵐTc-PMN (TLI 17.3 ± 1.5) in the
inflamed knees compared with the noninflamed knees.
In contrast, there was only a slight accumulation of
control IgG (TLI 1.2 ± 0.06). In contrast, anti-TNFα–
treated animals showed a significantly (P < 0.05) re-
duced tissue uptake of both anti–E-selectin MAb (TLI
9 ± 1) and PMN (TLI 8 ± 1) when compared with the
control animals. It should be noted, however, that
anti-TNFα did not fully abrogate either anti–E-selectin
uptake or PMN accumulation in the anti-TNFα–treated
group, in which case localization increments of 0 would
be achieved. There was no significant difference in the
Figure 5. Scintigraphic images of anti-E-selectin monoclonal antibody (MAb) uptake in untreated and anti-tumor necrosis factor α (anti-TNFα)-treated animals. Scintigraphic images of the hind limbs and abdomen of an untreated (A) and an anti-TNFα-treated (B) animal were taken 24 hours following the intraarticular injection of monosodium urate monohydrate crystals into the right knee and saline into the left knee. There is marked uptake of anti-E-selectin MAb into the inflamed knee of the untreated animal, particularly in the region of the joint space (A, arrow). In contrast, anti-E-selectin uptake in the injected knee of an anti-TNFα-treated animal demonstrates both a less intense and a less focal pattern of uptake (B, arrow).

Relative uptake of the control Ig between the 2 groups (TLI 1.2 ± 0.06 for the control group and 0.9 ± 0.2 for the anti-TNFα group).

Inflammatory skin lesions were induced by intradermal injection of IL-1α and TNFα at the time of induction of the arthritis. In anti-TNFα-treated animals,

Table 1. Uptake of 111In-labeled 1.2B6 F(ab')2 (anti-E-selectin monoclonal antibody), 99mTc-labeled polymorphonuclear cells (PMN), and 125I-labeled F(ab')2 MOPC 21 (control Ig) in synovium from control (untreated) and anti-tumor necrosis factor α (anti-TNFα)-treated pigs

<table>
<thead>
<tr>
<th>Marker, animal</th>
<th>Control animals</th>
<th>Anti-TNFα-treated animals</th>
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<tbody>
<tr>
<td></td>
<td>Inflamed knee, % ID/gm</td>
<td>Noninflamed knee, % ID/gm</td>
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<tr>
<td>1.2B6</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>0.06</td>
<td>0.0025</td>
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<td>3</td>
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<td>Total</td>
<td>0.05 ± 0.001‡</td>
<td>0.002 ± 0.0003</td>
</tr>
<tr>
<td>PMN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.014</td>
<td>0.0007</td>
</tr>
<tr>
<td>2</td>
<td>0.012</td>
<td>0.0007</td>
</tr>
<tr>
<td>3</td>
<td>0.011</td>
<td>0.0006</td>
</tr>
<tr>
<td>Total</td>
<td>0.012 ± 0.002</td>
<td>0.0007 ± 0.00006</td>
</tr>
<tr>
<td>MOPC 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0065</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.007</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>0.0065</td>
<td>0.003</td>
</tr>
<tr>
<td>Total</td>
<td>0.0067 ± 0.0003</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* Individual values are the mean of 3 synovial samples per animal. Totals are the mean ± SD of all 3 animals. % ID/gm = the percentage of injected dose per gram of tissue (synovium).

† The tissue localization increment (TLI) is a measure of the increased uptake in the inflamed knees (see Materials and Methods for formula).

‡ P < 0.05 compared with MOPC 21 control antibody (by Student’s paired t-test).

§ P < 0.05 compared with control animals (by Student’s paired t-test).
there was minimal anti–E-selectin uptake and PMN accumulation in the TNFα-induced sites; levels were comparable with those seen in noninjected and saline-injected skin. In contrast, there was significantly enhanced uptake of anti–E-selectin MAb and PMN accumulation in the IL-1α-treated sites (P < 0.05; data not shown). These data therefore suggest that the effects of anti-TNFα MAb were not due to nonspecific inhibition of EC activation or of PMN recruitment.

**DISCUSSION**

Over the last few years, there has been considerable interest in the roles of cytokines in mediating endothelial activation and leukocyte recruitment in inflammation. Despite abundant in vitro evidence for the important roles of TNFα and IL-1α/β in inducing endothelial activation, there is relatively little direct information on the relative contribution of these cytokines to endothelial activation in vivo. To address this question further, we have explored the cytokines involved in activating endothelium in a model of MSU crystal–induced inflammation, using a combined in vitro and in vivo approach in which E-selectin expression was measured as a marker of EC activation.

In the first part of this study, we used a bioassay together with a panel of neutralizing antibodies to determine which cytokines released by MSU crystal–activated MNC were responsible for inducing expression of adhesion molecules by cultured EC. We found that MSU crystals were a potent stimulus for IL-1 and TNFα production by monocytes, as previously reported (13,14,24–26). Although phagocytosis of MSU crystals by cultured EC has been shown to lead to superoxide anion generation (27), we were unable to demonstrate induced adhesion molecule expression following the direct incubation of MSU crystals with cultured EC. This suggests that up-regulation of adhesion molecule expression by endothelium in MSU crystal–induced inflammation in vivo is indirectly mediated following MSU crystal deposition.

We found that IL-1 and TNFα fully accounted for the E-selectin–inducing capacity in MSU crystal–stimulated monocyte supernatants, as shown by the complete loss of activity in the presence of a cocktail of MAb against IL-1α and IL-1β and TNFα and TNFβ. When supernatants were harvested 24 hours after incubation of MSU crystals with MNC, neither a combination of anti–IL-1α and anti–IL-1β, nor a combination of TNFα and TNFβ was able to inhibit endothelial-stimulating activity, indicating the presence of functional IL-1 and TNF. In contrast, a further experiment in which supernatants were harvested at earlier time points showed that the E-selectin–stimulating activity released by MNC in the first hour after exposure to MSU crystals was completely neutralized by anti–IL-1β. Whether IL-1 is necessary for subsequent TNF release in this model, or whether MSU crystals directly induce monocytes to release TNF with a more delayed kinetic than that of IL-1 remains to be determined. However, in preliminary experiments, it has not been possible to block TNF release by coincubating MNC with MSU crystals in the presence of anti–IL-1 antibodies (or vice versa).

Having established a role for TNFα in MSU crystal–induced, monocyte-mediated EC activation in vitro with both human and porcine EC, we turned our attention to the effects of inhibiting TNFα in vivo in MSU crystal–induced arthritis. Although anti-TNF alone failed to inhibit the in vitro stimulation of E-selectin by the supernatants of MNC exposed to MSU crystals, we found that TNFα blockade was very effective in suppressing both E-selectin expression and neutrophil emigration into inflamed joints, as judged by gamma-camera image analysis and postmortem tissue counting of radioactivity that had accumulated locally in synovium. This highlights the difficulty of modeling the complexities of cytokine networks in vivo with in vitro techniques. When the reagents become available for inhibiting IL-1 activity in vivo in the pig, it will obviously be interesting to compare the effect of inhibiting TNF with that of inhibiting IL-1 in our model. In view of our observation that, following exposure to MSU crystals, MNC appeared to release IL-1 before TNF, it will be particularly relevant to make this comparison at early time points after intraarticular injection of MSU crystals.

There is now a substantial body of literature regarding the inhibition of synovial inflammation in other animal models of arthritis, with reagents directed against either TNF or IL-1, using either antibodies, receptor antagonists, or recombinant receptors (for review, see ref. 28). Furthermore, the effects of inhibiting TNF and IL-1 have both been tested in the clinical setting (29–33). By focusing on E-selectin expression and neutrophil recruitment as endpoints, our in vivo study provides insights into the mechanisms by which anti-TNFα achieves therapeutic suppression of clinical inflammation. Our demonstration that anti-TNFα inhibits the local expression of E-selectin in inflamed joints is consistent with the reduction of expression of E-selectin in synovial biopsy tissues from rheumatoid arthritis patients receiving anti-TNFα (34).
The use of this radioisotopic MAb imaging technique for quantifying and localizing activated endothelium is not only useful for dissecting the pathophysiology of inflammation in vivo, but also may offer objective criteria for clinically assessing the components of inflammatory disease activity. This is particularly relevant in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, in which accurate assessment of local disease is difficult. With this in mind, we have recently performed a pilot imaging study with \( ^{111}\text{In}-\text{labeled anti-E-selectin MAb}\) 1.2B6 (F\(\text{[ab'}\)\(_2\)) in rheumatoid arthritis (35). We have been able to show that MAb 1.2B6 (F\(\text{[ab'}\)\(_2\)) provides distinct images of rheumatoid joints and is superior in terms of sensitivity, image intensity, and focal localization in comparison with \( ^{111}\text{In}-\text{labeled nonspecific human immunoglobulin}\).

It was of interest in the present study that imaging E-selectin enabled us to distinguish untreated from anti-TNF\(\alpha\)-treated animals, whereas no statistically significant distinction was obtained with radiolabeled leukocytes, another established clinical imaging technique.

We have shown in vitro that TNF\(\alpha\) and IL-1 are the principal mediators of MSU crystal-induced, monocyte-mediated endothelial activation, and that in vivo TNF\(\alpha\) blockade is effective in suppressing MSU crystal-induced arthritis. Of broader relevance is the demonstration that a neutralizing anti-TNF\(\alpha\) MAb achieves its inhibitory effect by inhibiting both synovial endothelial activation and neutrophil emigration into the joint. The present study also highlights the potential of anti-E-selectin imaging in the diagnosis and monitoring of inflammatory diseases in the clinical arena.

ACKNOWLEDGMENTS

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