Recruitment of Lymphocytes during Cutaneous Delayed Hypersensitivity in Nonhuman Primates Is Dependent on E-Selectin and Vascular Cell Adhesion Molecule 1

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Abstract

Previous investigations of cutaneous delayed hypersensitivity (DHR) in humans and animals have demonstrated that lymphocyte recruitment from blood is temporally and spatially associated with the de novo, asynchronous expression of both vascular cell adhesion molecule 1 (VCAM-1) and E-selectin on dermal endothelium. In this study, DHR was induced in rhesus monkeys sensitized against tuberculin in order to investigate the contribution of E-selectin and VCAM-1 in lymphocyte recruitment to skin. Intravenous infusions of neutralizing doses of $F(ab')_2$ fragments of murine antibodies to either E-selectin or VCAM-1 during the early inductive phases of DHR showed that murine IgG localized to dermal endothelium at the site of DHR in a pattern kinetically similar to the expression of each endothelial adhesion protein. Most importantly, the relative numbers of lymphocytes localized to the inflammatory site were significantly reduced in DHR modified with infusions of antibodies to either VCAM-1 or E-selectin, while the numbers of lymphocytes recruited to skin in the animal given $F(ab')_2$ fragments of an irrelevant murine monoclonal antibody of the same isotype and at the same dose were not changed. Moreover, in individual animals, the relative inhibition achieved with a particular antibody was proportional to the magnitude of expression of the targeted adhesion protein. Therefore, both VCAM-1 and E-selectin are functionally relevant in the genesis of cutaneous DHR, and each appears to contribute to lymphocyte recruitment in relation to its relative degree of expression in any one particular animal. (*J. Clin. Invest.* 1994. 93:1554–1563.) Key words: rhesus monkeys • skin, endothelium • adhesion • T cell

Introduction

There is considerable evidence that leukocyte and endothelial adhesion molecules play pivotal roles in the recruitment of leukocytes to sites of inflammatory reactions. Adhesion proteins on endothelium, such as P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1),[1] or intercellular adhesion molecule 1 (ICAM-1) are either expressed *de novo* (VCAM-1, E-selectin), redistributed to the cell membrane from cytoplasmic stores (P-selectin), or upregulated from basal levels (ICAM-1) in multiple organs with various kinds of inflammatory disease activity, including inflammatory bowel disease (1, 2), sepsis (3), AIDS encephalitis in monkeys (4), complement-mediated pulmonary injury in rats (5), allograft rejection (6, 7), and cutaneous delayed hypersensitivity (DHR) (8–12). In experimental models of inflammatory disease, there are close spatial and temporal associations between the expression of endothelial adhesion molecules and the subsequent localization of leukocytes at the inflammatory site (9–16). However, because multiple adhesion proteins are usually expressed at inflammatory sites and are likely acting in concert, the relative contribution of specific endothelial adhesion proteins to leukocyte recruitment cannot be assessed using descriptive studies alone.

The use of in vivo-administered murine mAbs to specific leukocyte and endothelial adhesion proteins in various animal models has provided valuable information about the role of specific adhesion proteins in the evolving inflammatory response. When administered to rabbits, mAbs to the common β chain (CD18) of β2-integrins on leukocytes significantly reduced PMN recruitment in acute inflammatory models (17–20). Similarly, mAbs that inhibit β2-integrin interactions with endothelial ICAM-1, when administered to rats, reduced PMN recruitment, vascular permeability, or hemorrhage in various acute inflammatory settings (21–23). Moreover, in the same or similar rat or murine acute inflammatory models, blockades of endothelial E- or P-selectin or leukocyte L-selectin in vivo were found to be efficacious in reducing inflammatory sequelae (5, 24–27). Therefore, experimental evidence in animal models suggests that inflammatory disease activity, at least in inflammatory reactions characterized by the presence of PMNs, can be modified by interfering with leukocyte-endothelial adhesive interactions.

In contrast to the work with PMN-mediated inflammatory disease in acute inflammatory models, there is significantly less known about the adhesion molecules utilized for the recruitment of lymphocytes and macrophages in chronic inflammatory settings, primarily because the administration of mAbs for extended periods of time in animal models is usually not clinically feasible or efficacious. Nevertheless, mAbs against the β1-

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Received for publication 25 October 1993 and in revised form 22 December 1993.

J. Clin. Invest.

0021-9738/94/04/1554/10 $2.00
Volume 93, April 1994, 1554–1563

1. *Abbreviations used in this paper:* DHR, delayed hypersensitivity; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; RUVEC, rhesus monkey umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

integrin, very late antigen 4 (VLA-4; $\alpha 4\beta 1$-integrin), in rats inhibited lymphocyte recruitment to joints during adjuvant-induced arthritis (28), to central nervous system (CNS) during experimental allergic encephalomyelitis (29), and to skin after injection of proinflammatory cytokines or during DHR (30). In addition, mAbs to ICAM-1 reduced lymphocyte localization in renal allografts and prolonged graft survival in nonhuman primates (6). However, the relative contribution of various endothelial adhesion proteins in an inflammatory reaction characterized by lymphocytic infiltration has not, to our knowledge, been examined.

Recently, using immunohistochemistry, we characterized cutaneous DHR against tuberculin in a nonhuman primate model and found an asynchronous expression of both E-selectin and VCAM-1 on dermal endothelium (9). The contribution of each in lymphocyte recruitment could not be assessed. In this study, we administered relevant neutralizing mAbs to E-selectin and VCAM-1 in vivo during the early inductive phase of DHR in this nonhuman primate model. We found that functional expression of both E-selectin and VCAM-1 is requisite for the genesis of lymphocytic infiltration in cutaneous DHR and that blockade efficacy in any one animal is proportional to the relative degree of expression of the targeted adhesion protein.

Methods

Animals and antibodies. Cutaneous DHR was induced at several sites on the backs of five healthy adult rhesus monkeys by intradermal administration of 0.1 ml of tuberculin made from standardized filtrates from cultures of *Mycobacterium tuberculosis* (Coopers Animal Health Inc., Kansas City, KS; lots KC166R and KC167R). These five animals were previously sensitized to *M. tuberculosis* by the intradermal injection of CFA 9–52 mo before the study. Serum and skin injection sites were obtained by venipuncture and surgical biopsy at 0, 4, 8, 24, 48, 72, 96, 144, 168, and 264 h after injection. Each injection site was biopsied only once. These animals were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals, National Research Council.

Cutaneous DHR was induced in each of the five macaques at least once before administration of mAb therapy in order to compare unmodified DHR with DHR during mAb therapy. For the blockade of E-selectin in vivo, two animals (346-80 and 152-81) were administered $F(ab')_2$ fragments of murine mAb 7A9 (IgG1) (31, 32) intravenously. For the blockade of VCAM-1 in vivo, $F(ab')_2$ fragments of murine mAb 2G7 (IgG1) (4, 32–34) were administered intravenously to two other animals (233-86 and 300-73). $F(ab')_2$ fragments were prepared as previously described (32) and were utilized in order to avoid any potential bridging of leukocytes and endothelium via Fc receptors on the former. Both 7A9 and 2G7 $F(ab')_2$ fragments have been previously shown to recognize human E-selectin and VCAM-1, respectively, and maximally inhibit human leukocyte cell binding to IL-1–stimulated human umbilical vein endothelial cells (HUVEC) in vitro at concentrations of 2–10 μg/ml (31, 32). In addition, both antibodies recognize the homologous rhesus monkey epitope (4, 9, 34, 35). The dosing regime for each antibody in macaques was determined from pharmacokinetic profiles of single-dose intravenous injections of each antibody ($F(ab')_2$ fragments) at 2.0 mg/kg in four additional macaque monkeys. These studies demonstrated that the approximate half-life of 7A9 was 12 h whereas that of 2G7 was 5 h (data not shown). From these data, maintenance of serum neutralizing concentrations of each antibody (10 μg/ml) was projected using an initial loading dose of each antibody of 2.0 mg/kg intravenously, followed by maintenance doses of 1.0 mg/kg intravenously every 12 h. mAb infusions were performed during the first 96 h of DHR. As a control, one animal (animal 55-84) received $F(ab')_2$ fragments of mAb 5E9 (36, 37) (kindly provided by Drs. Walter Blattler and John Lambert, Immunogen Inc., Cambridge, MA) at the same dosing strategy. mAb 5E9 recognizes the human transferrin receptor (36) yet does not cross-react with the rhesus monkey epitope based on flow cytometry and immunohistochemical techniques (data not shown; Drs. A. Silber and D. Ringler, personal observation).

To determine if desensitization to tuberculin occurred in the study animals upon multiple exposures to intradermal tuberculin, two animals underwent a third DHR after mAb treatment.

Rhesus endothelial cell binding assay. Rhesus monkey umbilical vein endothelial cells (RUVEC) were harvested as previously described (38), and were maintained in M199 medium (Whittaker Bioproducts, Walkersville, MD) containing 20% endotoxin-tested fetal bovine serum (Gibco Laboratories, Grand Island, NY), 90 μg/ml preservative-free porcine heparin (Sigma Chemical Co., St. Louis, MO), 100 μg/ml bovine hypothalamus extract (Otsuka America Pharmaceutical Inc., Rockville, MD), 50 U/ml of penicillin, 50 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (Gibco Laboratories). Cells were grown to confluence in gelatin-coated 75-cm^2 polystyrene flasks and were passaged by dislodgment with washes of 2.5 g/liter trypsin (Sigma Chemical Co.) and calcium- and magnesium-free Hanks balanced salt solution (HBSS) (Gibco Laboratories) and reseeding onto other gelatin-coated flasks or flat-bottomed 24-well or 96-well plates.

In order to assess the neutralization potential of 7A9 $F(ab')_2$ mAb to inhibit leukocyte binding to rhesus monkey E-selectin, adhesion of primary ^{51}Cr-labeled human neutrophils to resting and cytokine-stimulated RUVEC was evaluated, as previously described using HUVEC (32). Briefly, human neutrophils were obtained from heparinized peripheral blood from which the mononuclear cells had been removed by centrifugation over Ficoll-Hypaque (Sigma Chemical Co.). The neutrophil/red cell pellet was sedimented over 3% dextran to remove the majority of red cells. Remaining red cells, when necessary, were lysed with hypotonic saline. Purity of neutrophils was > 95%, as determined by Wright-Giemsa staining. RUVEC, at passage 9 or less, were plated onto 24-well plates precoated with gelatin and cultured for 48 h or until confluent. Some cultures were exposed to 1.0 ng/ml of human rIL-1β (generous gift of Dr. Y. Hirai, Tokushima Research Institute, Tokushima, Japan) for 4 h at 37°C. After rinsing, monolayers were exposed to various concentrations of $F(ab')_2$ fragments of 7A9 antibody in RPMI 1640 containing 10% FCS for 30 min at 37°C. Control wells contained supplemented medium only or 10 μg/ml of the purified myeloma protein, MOPC-21. All experimental points were done in triplicate. Monolayers were overlaid, in the presence of antibody, with $\sim 10^6$ ^{51}Cr-labeled neutrophils in a volume of 0.5 ml. After 45 min at 37°C, monolayers were gently rinsed twice with wash medium at room temperature. The remaining adherent target cells were lysed with 300 μl of 1% Triton X-100 in PBS. One half of this volume (150 μl) was counted in a gamma counter. The amount of ^{51}Cr taken up by 10^6 washed target cells was used to convert data from cpm/well to number of cells bound/well.

In order to assess the neutralization potential of 2G7 mAb to inhibit leukocyte binding to rhesus monkey VCAM-1, a similar adhesion assay using RUVEC was used, except that adhesion of a human monocytic cell line, THP-1 (ATCC No. TIB 202, Rockville, MD), to resting and TNF-α-stimulated RUVEC was assessed, and the method of counting bound cells was modified. Briefly, RUVEC (< 9th passage) were first plated onto gelatin-coated flat-bottomed 96-well plates for $\sim$ 48 h. Some cultures were subsequently stimulated with 10 ng/ml human recombinant TNF-α (Sigma Chemical Co.) for 4 h followed by rinsing in supplemented medium. As described above using 7A9 mAb, RUVEC monolayers were incubated with $F(ab')_2$ fragments of 2G7 mAb diluted in supplemented RPMI medium for 30 min at 37°C. Control wells contained supplemented medium only or $F(ab')_2$ fragments of the irrelevant IgG1 antibody, 5E9. Next, $\sim 0.5 \times 10^6$ cells were added to each well and incubated with RUVEC for 30 min at 37°C. After incubation, nonadherent cells were removed from wells by

two consecutive washes with complete medium and placed in another 96-well plate and counted. The number of nonadherent viable cells was determined by a colorimetric assay based on the ability of live cells to reduce a tetrazolium-based compound (MTT) (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to a blue formazan product (39). Briefly, 0.5 mg/ml of MTT was added to the nonadherent cell fraction for 4 h at 37°C, during which mitochondrial dehydrogenase from the nonadherent cells reduced MTT, resulting in insoluble formazan crystals. The subsequent addition of 150 μl/well of anhydrous isopropanol yielded a colorimetric change proportionally related to the quantity of mitochondrial dehydrogenase and resultant formazan crystals. The number of nonadherent cells was then determined from a standard curve of optical density versus cell number, and the number of adherent cells was calculated by subtraction. Consistent results were obtained using RUVEC passaged less than nine times.

Percent inhibition of binding of target cells by both antibodies was calculated as follows: [(number of bound cells after cytokine minus number of bound cells after mAb and cytokine)/(number of bound cells after cytokine)] ×100.

Tissues. Skin samples were divided so that half were fixed in 10% buffered formalin for histology and half embedded in OCT compound (Miles Inc., Elkhart, IN), snap-frozen in 2-methylbutane cooled in dry ice, and stored at −80°C for subsequent immunohistologic techniques. Formalin-fixed skin samples were embedded in paraffin and sections stained with hematoxylin and eosin for routine histologic examination. Immunohistochemistry was performed on desiccated 7.0-μm frozen skin sections mounted on gelatin-coated glass slides. Briefly, tissue sections were fixed in 2% paraformaldehyde/0.5 × PBS (pH 7.2) for 10 min at 4°C. To localize and quantify the number of CD2+ lymphocytes in skin tissue sections, an avidin-biotin-horseradish peroxidase complex technique was utilized, using the mAb, T11/3Pt2H9 (Coulter Immunology, Hialeah, FL) as the primary antibody and diaminobenzidine (Sigma Chemical Co.) as the chromogen, as previously described (40–42). Previous experiments demonstrated that the mAb T11/3Pt2H9 recognizes the rhesus monkey CD2 determinant (9, 40, 41). Immunohistochemistry for E-selectin and VCAM-1 expression in skin was performed using skin sections immunostained using 3B7, a murine antibody to human E-selectin, and 2G7 or 1E7, murine antibodies to human VCAM-1 (31, 32). All three antibodies have been shown previously to recognize the homologous E-selectin or VCAM-1 epitope in rhesus macaques (4, 9, 34). As negative controls, step sections of each tissue sample were processed under identical conditions with the exception that the primary antibody was substituted with an irrelevant murine antibody of the same isotype and concentration. To localize murine $F(ab')_2$ mAbs in skin tissue sections after in vivo administration, the primary antibody was omitted and a biotinylated goat anti-mouse IgG ($F(ab')_2$ fragment-specific) (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody in the sequence. To localize CD2 for lymphocyte quantification in skin from animals receiving murine $F(ab')_2$ mAbs, whole T11/3Pt2H9 was used as the primary antibody and a biotinylated goat anti-mouse IgG (Fc fragment-specific) (Jackson ImmunoResearch Laboratories) was used as the secondary antibody in the sequence.

Semiquantitative immunohistochemical analysis. The numbers of CD2+ lymphocytes in skin tissue sections were assessed semiquantitatively using a scoring system previously described (9) and modified from that used by Munro et al. (13, 14) and Briscoe et al. (15). This scoring method is based on both the extent and composition of leukocyte infiltration as criteria (9). Relative infiltration was judged using hematoxylin and eosin–stained histologic slides and the scores: 0, normal; 1, small perivascular infiltrates (< 5× normal size) or increased numbers of scattered leukocytes in the dermis; 2, small perivascular infiltrates and increased scattered leukocytes in the dermis; 3, large perivascular infiltrates (> 5× normal size); or 4, lichenoid infiltration. Composition of infiltrates was then assessed using immunohistochemistry for CD2, and assigned scores for the percentage of the infiltrate that was immunoreactive included: 0, no cells immunoreactive; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%. The final score was the product of the values obtained from these two criteria and had a theoretical range from 0 to 16. Each sample was evaluated blindly by two observers (Drs. Ringler and Silber), and mean scores±1 SEM of animals receiving the same antibody infusions were plotted against time after initiation of DHR and compared to mean scores±1 SEM of the same animals in a previous unmodified DHR. Each animal responded comparably in lymphocyte infiltration scores when examined in multiple subsequent unmodified cutaneous reactions, both before and after antibody treatment.

The kinetic expression of the adhesion molecules VCAM-1 and E-selectin was characterized semiquantitatively using a similar scoring system as previously described (9). Briefly, endothelial expression of adhesion molecules was evaluated using both numbers of immunoreactive dermal vessels in a 0.5-cm linear biopsy sample and intensity of staining as criteria. For relative numbers of immunoreactive vessels, assigned scores included: 0, no vessels; 1, 1–4 vessels; 2, 5–10 vessels; 3, 11–15 vessels; 4, 16–20 vessels; and 5, > 20 vessels. The scores for staining intensity were: 1, faint; 2, moderate, with or without 4 vessels being intense; and 3, intense staining of > 4 dermal vessels. The product of these two values for intensity and numbers of immunoreactive vessels was then used as the final assigned score, which had a theoretical range from 0 to 15. Each sample was scored without knowledge of the identity of the animal by two of us and means established, as described above.

Quantification of murine IgG in monkey serum. Levels of antibody in serum were determined by a sandwich ELISA. The assay was done by coating Nunc 96-well ELISA plates with 100 μl of goat anti–mouse IgG(H + L) (Jackson ImmunoResearch Laboratories) at 2.5 μg/ml in 0.1 M Na carbonate buffer, pH 9.0, overnight at 4°C. Plates were washed with PBS/Tween 0.05% and blocked for 1 h at 37°C with PBS/1% bovine serum albumin (Sigma Chemical Co.). The antibody standard for the assay was the same used for the experiment. Standard at 50 ng/ml in PBS/1% BSA was added to the first well and serial twofold dilutions in PBS/1% BSA were done across the plate. Monkey serum samples were initially diluted 1:25, and 200 μl was added to the plate, with subsequent serial twofold dilutions. Plates were incubated for 2 h at 25°C, washed, and 100 μl of peroxidase-conjugated goat anti–mouse IgG(H + L) (Jackson ImmunoResearch Laboratories) at 375 ng/ml in PBS/1% BSA added. Plates were incubated an additional 2 h at 25°C. Plates were then washed followed by the addition of *O*-phenylenediamine (1 mg/ml) (Zymed Laboratories, San Francisco, CA) in phosphate citrate buffer and 0.03% hydrogen peroxide. After shaking for 15 minutes in the dark, the reaction was stopped with the addition of 12.5% sulfuric acid and read at absorbance 492 nm. Linear regression analysis of the standards was performed and antibody levels calculated. The R-squared value for all assays was at least 0.98, and triplicate determinations of antibody levels were done on each sample. The limit of detection for the assay was ~ 150 pg/ml.

Statistics. Significance was determined using the paired Student's *t* test. Differences between means were considered significant when $P < 0.05$.

Results

mAb cross-Reactivity to rhesus monkey epitopes. Both 7A9 mAb (anti–E-selectin) and 2G7 (anti-VCAM-1) have been previously shown to recognize homologous rhesus monkey determinants in tissue sections using immunohistochemistry (9) and to inhibit binding of human leukocytes to cytokine-stimulated HUVEC in vitro (32). Furthermore, 2G7 has been shown to inhibit VCAM-1-mediated adhesion of human monocytic cell lines to rhesus monkey endothelium using the Stamper-Woodruff tissue adhesion assay (34). Nevertheless, before infusion into rhesus monkeys, both antibodies were examined for their ability to inhibit adhesion of leukocytes to rhesus monkey E-selectin and VCAM-1, respectively, using

Table I. Inhibition of Adhesion of Human Neutrophils and THP-1 Cells to Cytokine-stimulated RUVEC

	RUVEC	HUVEC
	percent cells bound	
Granulocytes		
IL-1 treatment	31	20
IL-1 and 7A9 mAb	10 (68% inhibition)	9 (55% inhibition)
THP-1 cells		
TNF-α treatment	52	NP
TNF-α and 2G7 mAb	34 (36% inhibition)	NP

Data are those from a single representative experiment. RUVEC were stimulated with human rIL-1 (1.0 ng/ml) or TNF-α (10 ng/ml) for 4 h followed by pretreatment with and without $F(ab')_2$ mAbs to E-selectin (7A9) (10 μg/ml) or VCAM-1 (2G7) (20 μg/ml), as described in Methods. Granulocytes were used to evaluate E-selectin-mediated adhesion to RUVEC, while THP-1 cells were used to evaluate VCAM-1-mediated binding. Abbreviation: NP, not performed.

RUVEC. For these experiments, RUVEC were stimulated with either human rIL-1 or rTNF-α, exposed to various concentrations of $F(ab')_2$ fragments of 7A9 or 2G7, and subsequently examined for changes in adhesive potential for human leukocytes or leukocyte cell lines. As seen in Table I, pretreatment of RUVEC with $F(ab')_2$ fragments of 7A9 inhibited binding of human neutrophils by as much as 68% compared to no treatment, while pretreatment of RUVEC with $F(ab')_2$ fragments of 2G7 inhibited adhesion of human monocytic cells by ~ 36%. These values compare favorably to those previously reported for E-selectin-mediated binding of human neutrophils or VCAM-1-mediated adhesion of mononuclear cells for HUVEC (32). Maximal inhibition was obtained at mAb concentrations from 2 to 10 μg/ml (data not shown), similar to previous observations using HUVEC (32).

mAb infusions. In the two animals receiving infusions of the anti-VCAM-1 mAb, 2G7, pre- and postinfusion (5 min) concentrations of murine IgG in serum ranged from 14.2 to 181.1 μg/ml, respectively (Fig. 1). At 120 h (5 d) after initiation of DHR and 24 h after the last infusion of 2G7, concentrations of murine IgG in both animals were < 8.0 μg/ml. By 144 h (6 d) and beyond, serum concentrations of murine IgG in both animals were < 1.0 μg/ml. In the two animals receiving 7A9, serum concentrations of murine IgG ranged from 22.2 to 122.4 μg/ml (Fig. 1). Consistent with the longer half-life of 7A9, by 120 h (5 d), both animals still had 7A9 serum concentrations > 15.0 μg/ml, levels consistent with an effective E-selectin blockade using RUVEC in vitro. However, by 144 h (6 d), animal 346-80 had < 1.0 μg/ml of 7A9 immunoglobulin in serum, while animal 152-81 had a significantly higher (16.6 μg/ml) serum concentration of murine IgG. By 168 h (7 d), the last time point examined, murine IgG could not be detected in animal 346-80, while animal 152-81 had ~ 7.3 μg/ml of murine IgG in the serum. Thus, despite a consistently longer half-life for 7A9 antibody in vivo than 2G7, there was significant variation in 7A9 serum concentrations between study animals.

To determine if infused mAb localized to endothelium at the inflammatory site, immunohistochemical procedures were performed for the localization of $F(ab')_2$ murine IgG. As expected, in animals receiving either 7A9 or 2G7, murine IgG could be detected on superficial and deep dermal venules and rare arterioles within the inflammatory sites (Fig. 2, *C* and *F*)

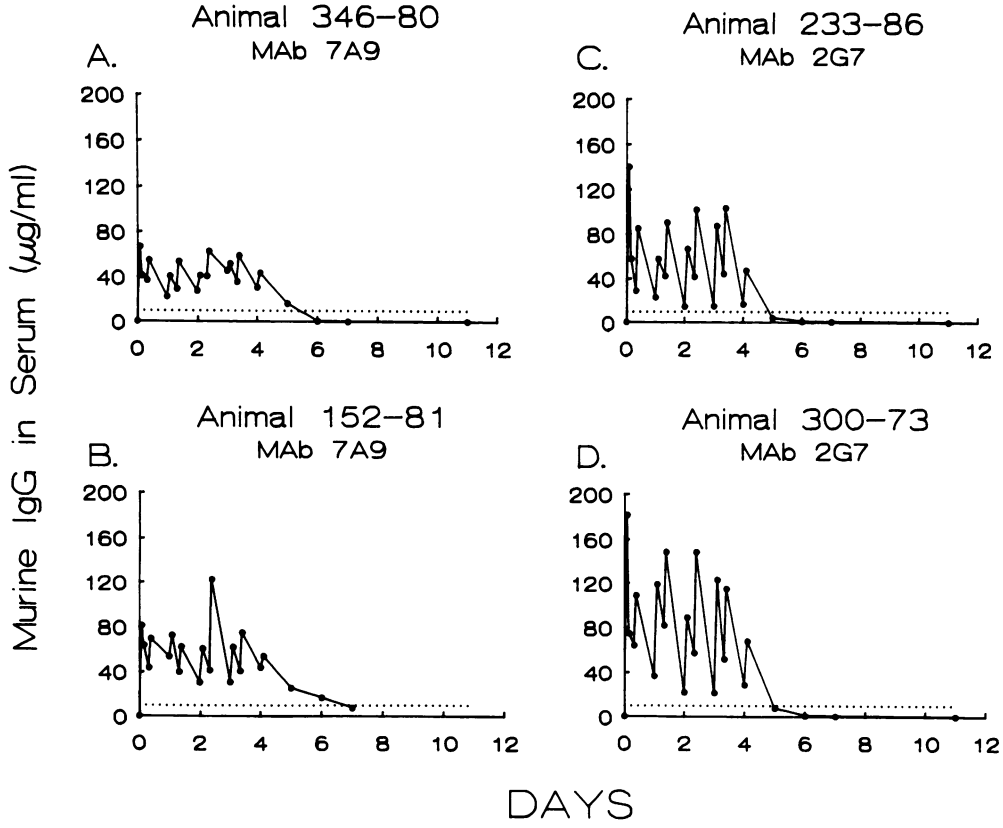

Figure 1. Murine IgG concentrations in serum from the study animals during DHR. (*A* and *B*) In animals (346-80 and 152-81) receiving anti-E-selectin antibody, 7A9, serum concentrations of IgG were above 10 μg/ml (*horizontal dotted line*) from initiation of DHR until day 6. (*C* and *D*) In animals (233-86 and 300-73) receiving anti-VCAM-1 antibodies, serum concentrations of IgG were above 10 μg/ml from initiation until day 5.

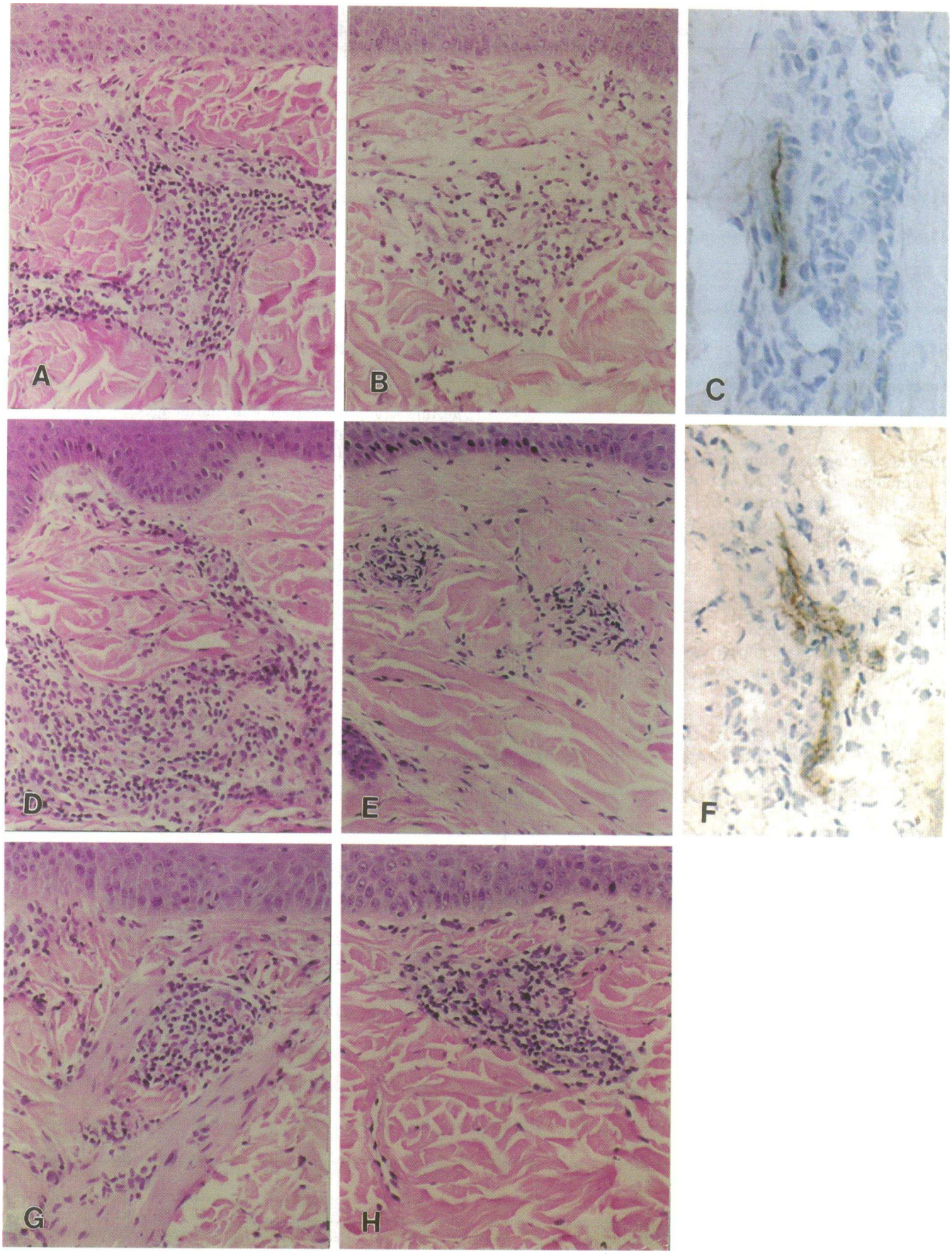

Figure 2. Cutaneous DHR in the rhesus monkey with and without in vivo administration of antibody to endothelial adhesion molecules. (*A–C*) In animal 233-86, unmodified DHR at day 4 (*A*) is characterized by dense angiocentric lymphocytic infiltrates in the superficial dermis. In

as early as 4 h after induction of DHR for 7A9 and 8 h for 2G7, consistent with the kinetic expression of E-selectin and VCAM-1, respectively, in these animals, as reported previously (9). This reactivity persisted in all four animals through day 6, a time period when the serum concentrations of mAb in three of the animals were < 1.0 μg/ml. However, at day 6, the staining intensity and number of immunoreactive dermal venules were considerably less than at previous time points. By day 7, murine IgG could not be localized to any skin sample. Murine IgG could not be localized at any time period in skin samples from the animal receiving the irrelevant mAb, 5E9. Collectively, these data suggest that neutralizing concentrations of 7A9 and 2G7 were achieved in the serum in all four animals through day 5 of the inflammatory reaction and that these mAbs localized to endothelium at the inflammatory site through day 6.

Clinical efficacy. We next examined if infusions of either anti-E-selectin or anti-VCAM-1 antibodies modified the inflammatory response. Gross examination of the injection sites revealed no appreciable or consistent difference in the size or intensity of erythema at the inflammatory site in untreated DHR versus DHR modified by mAb therapy, either with 7A9 or 2G7 antibodies. However, on histological examination of skin during the inflammatory course, there was a significant reduction in the number of lymphocytes recruited to the inflammatory site in DHR modified with either anti-E-selectin (7A9) or anti-VCAM-1 (2G7) mAbs, yet the time at which this difference was observed varied for each antibody (Figs. 2 and 3). When compared to a previous unmodified DHR, in the two animals receiving anti-VCAM-1 mAb 2G7, there was a trend for reduced lymphocyte recruitment at 24 h and beyond; however, this difference was significant only at day 4 ($P < 0.01$; Figs. 2, *A* and *B*, and 3). Similarly, in the animals receiving anti-E-selectin mAb 7A9, there was a diminution of lymphocyte recruitment at most time points examined (Fig. 3). However, in contrast to that seen with anti-VCAM-1 immunotherapy, anti-E-selectin mAb infusions statistically reduced local lymphocyte localization at early (day 2) and late (days 6 and 7) time points in the inflammatory cascade ($P < 0.05$; Figs. 2, *D* and *E*, and 3). Furthermore, in both groups of animals receiving mAbs, in addition to a reduction in the size of the perivascular infiltrates, there appeared to be more interstitial edema and a reduction in cellular density within the infiltrates (Fig. 2 *B*). In the animal receiving irrelevant mAb, there was no apparent difference in the numbers of CD2+ cells at the injection site when compared to DHR in the same animal without mAb infusion (Figs. 2, *G* and *H*, and 3). In subsequent DHR after mAb infusions in two of the study animals, there was no difference in the numbers of CD2+ lymphocytes at inflammatory sites compared to earlier DHR (data not shown). Therefore, animals did not become desensitized to tuberculin after multiple intradermal exposures.

In an effort to examine the mechanisms associated with outcome from mAb immunotherapy, the relative expression of

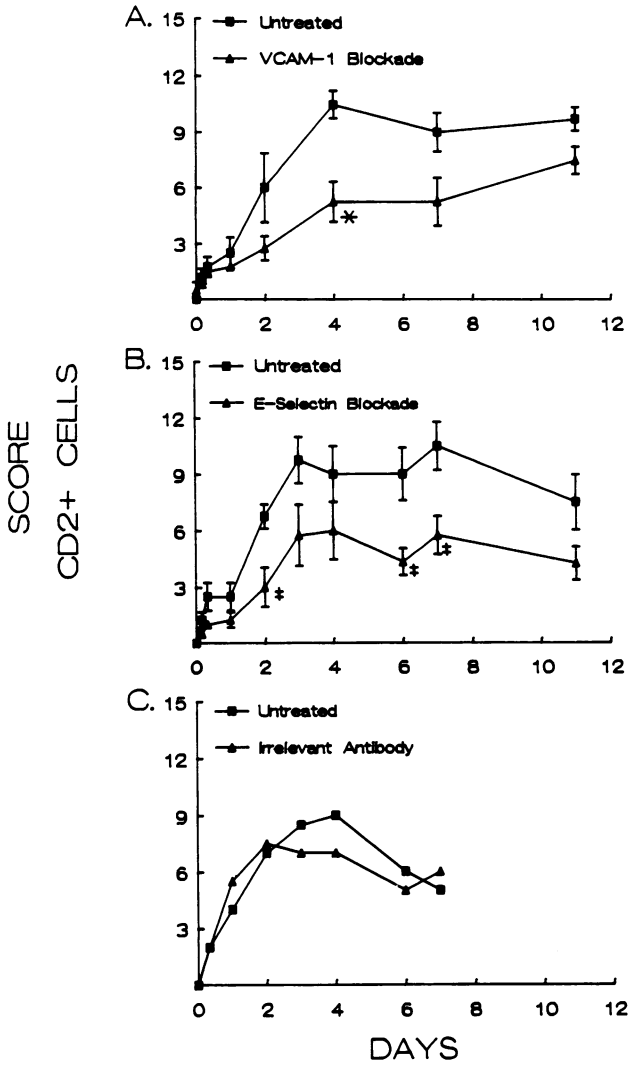

Figure 3. Relative temporal scores (mean±1 SEM) for the numbers of CD2+ lymphocytes in treated (— ▲ —) and untreated (— ■ —) DHR in animals receiving mAbs 2G7 (anti-VCAM-1) (*A*), 7A9 (anti-E-selectin) (*B*), and 5E9 (irrelevant antibody) (*C*). Significant reductions in the numbers of skin T cells were seen in animals given mAbs to VCAM-1 (day 4; $P < 0.01$) and E-selectin (days *2, 6,* and *7;* $P < 0.05$), yet comparable reductions were not observed in the animal given irrelevant murine antibody. Subscripts at the time points indicate that the values are significantly different from the untreated control group. $^{*}P < 0.01$, $^{‡}P < 0.05$.

E-selectin and VCAM-1 was compared to the degree by which 7A9 or 2G7 mAbs were efficacious in a subsequent DHR in reducing lymphocyte localization in a specific animal. VCAM-1 expression on dermal endothelium was similar in the two animals receiving 2G7, and both animals had comparable reductions in the numbers of lymphocytes at the inflammatory site during mAb administration (Fig. 4). However, in the two

DHR at day 4 modified by in vivo administration of anti-VCAM-1 antibodies (*B*), lymphocytic infiltrates are edematous, contain fewer lymphocytes, and are typically smaller (not shown). During this VCAM-1 blockade, murine IgG can be localized to dermal venules (*C*, day 3). (*D–F*) In animal 152-81, infusion of antibodies to E-selectin in vivo during DHR (*E*) resulted in significant reduction in the size of perivascular infiltrates at day 6 compared to unmodified DHR (*D*). During this E-selectin blockade, murine IgG can be localized to dermal venules (*F*, day 2). (*G* and *H*) In contrast, irrelevant murine antibodies given to animal 55-84 (*H*) did not change the character of lymphocytic infiltration during DHR at day 3 compared to DHR without mAb administration (*G*). (*A, B, D, E, G,* and *H*) × 180, hematoxylin and eosin; (*C* and *F*) × 360, immunoperoxidase technique with Mayer's hematoxylin counterstain).

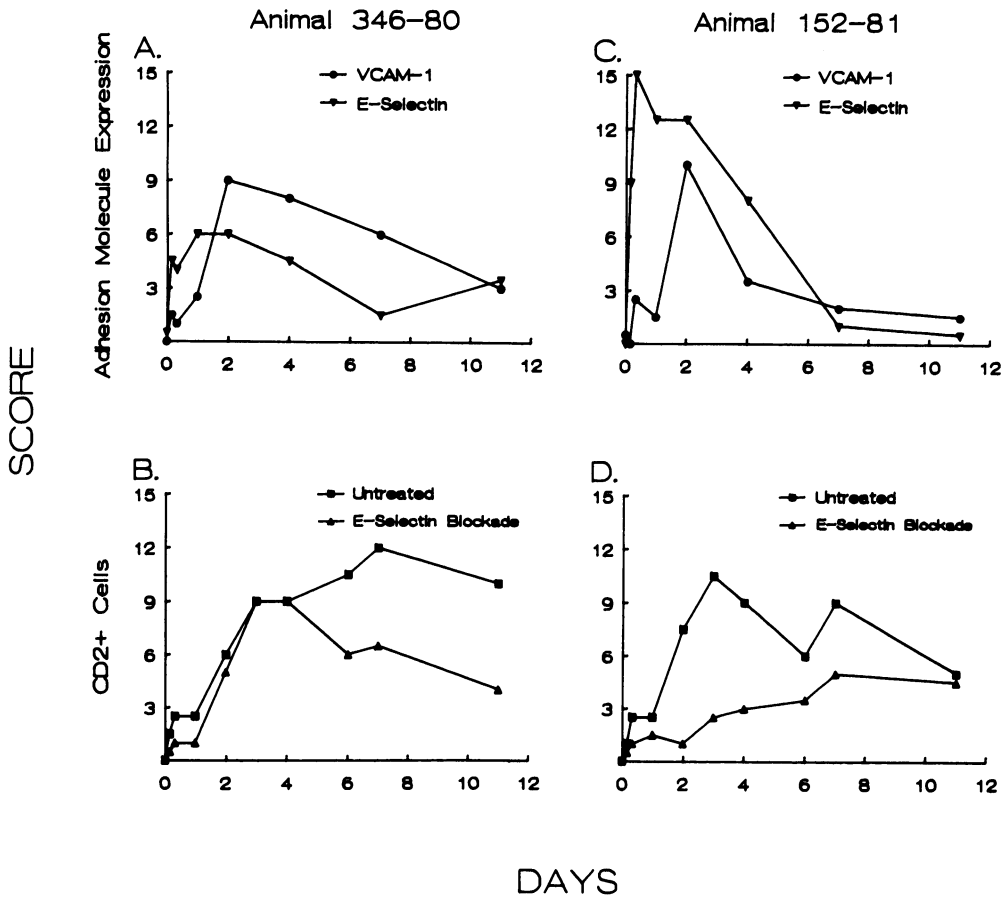

Figure 4. Individual scores for E-selectin (— ▼ —) and VCAM-1 (— ● —) expression in untreated DHR in animals 346-80 and 152-81 (*A* and *C*) and relative scores for the numbers of CD2+ lymphocytes with (— ▲ —) and without (— ■ —) infusions of mAb to E-selectin in the same animals (*B* and *D*). While both animals had comparable levels of VCAM-1 immunoreactivity in untreated DHR (*A* and *C*), animal 152-81 had approximately twice the score for E-selectin expression during the first 2 d than did animal 346-80 (*A* and *C*). When antibodies to E-selectin were administered (*B* and *D*), a significant diminution of the numbers of skin CD2+ T cells was observed in animal 152-81 at all time points examined, with the most noticeable reductions seen at days 2–4 (*D*). In contrast, comparable reductions in the numbers of skin CD2+ T cells at days 2–4 were not observed in animal 346-80 during DHR modified with infusions of antibodies to E-selectin (*B*).

animals receiving anti-E-selectin mAb, 7A9, there were dramatic differences in E-selectin expression on dermal endothelium (Fig. 4). In the animal (152-81) with the highest E-selectin expression, 7A9 mAb infusions were associated with the largest reduction in the number of cutaneous lymphocytes, predominantly from 8 h up to 11 days after DHR induction (Fig. 4). In contrast, in the other animal (346-80) receiving 7A9 mAb, E-selectin expression was approximately half that seen in animal 152-81, and attenuation in the numbers of lymphocytes recruited at the site was not seen until day 6 and beyond (Fig. 4). Therefore, the outcome derived from an in vivo E-selectin blockade in DHR, at least in terms of lymphocyte recruitment, was associated with the relative extent of E-selectin expression on dermal endothelium.

Discussion

Because we and others have previously demonstrated that cutaneous DHR is characterized by the dual, asynchronous, *de novo* expression of E-selectin and VCAM-1 (8–10, 12, 43), the purpose of this study was to examine the contribution of each endothelial adhesion protein in the genesis of clinically apparent cutaneous DHR by infusing neutralizing doses of murine mAbs to each protein in a relevant animal model. We demonstrate that: (*a*) blocking mAbs to either E-selectin or VCAM-1, when administered to rhesus monkeys, localize to endothelium at sites of cutaneous DHR; (*b*) localization of both mAbs at cutaneous sites is spatially and temporally similar to the expression of E-selectin and VCAM-1; (*c*) localization of either mAb at the inflammatory site is associated with a significant reduction in the numbers of dermal lymphocytes within angiocentric infiltrates; and (*d*) the relative inhibition of lymphocyte recruitment is associated with the magnitude of expression of the targeted adhesion molecule.

It has been previously shown that specific endothelial adhesion proteins and their respective leukocyte ligands can mediate adhesion of leukocytes in vitro (31, 32, 44–54) and that these endothelial adhesion proteins are expressed at inflammatory sites (1–7, 9–12). Although it has been hypothesized from these data that endothelial and leukocyte adhesion molecules play a fundamental role in leukocyte recruitment and subsequent tissue injury at an inflammatory site, only recently, using neutralizing mAbs and animal models of inflammatory disease, has this role been documented. mAbs to integrins (either CD18, CD11a, or CD11b) or selectins, when administered to either rodents or rabbits during an acute inflammatory response, reduced PMN recruitment to the inflammatory site (17–20, 26, 27) and often attenuated endothelial injury, as assessed by hemorrhage and/or edema (5, 21–23, 25). Because adhesion of PMNs to endothelium can result in significant PMN-mediated endothelial injury (55), it follows that inhibition of adhesion of PMNs to endothelium at inflammatory sites can have clinically beneficial effects. Significantly less experimental work has focused on mononuclear leukocyte adhesion in chronic inflammatory disease, yet administration of mAbs against the β1 integrin, VLA-4 ($\alpha 4\beta 1$-integrin), in rats

inhibited lymphocyte recruitment in a number of inflammatory settings (28–30), and mAbs to ICAM-1 given to nonhuman primates reduced lymphocytic infiltration in renal allografts and prolonged graft survival (6). With regard to the role of VCAM-1/VLA-4 interactions in lymphocyte recruitment at an inflammatory site, our data contribute to the findings described in rats for EAE (29), adjuvant-induced arthritis (28), and cutaneous inflammation (30). Moreover, our data extend these observations by demonstrating, for the first time, that inhibition of lymphocytic recruitment in vivo can be accomplished by mAb therapy directed against either endothelial E-selectin or VCAM-1.

In our study using nonhuman primates, blockades directed against E-selectin or VCAM-1 did not appear to ameliorate the vascular permeability associated with the inflammatory response, as significant perivascular dermal edema could be visualized histologically in samples from animals receiving mAbs to either endothelial protein. Although increased vascular permeability may theoretically arise from endothelial injury secondary to murine antibody localization on the membrane, there are a number of reasons that argue against this notion. First, this edema is not likely secondary to endothelial injury mediated by antibody-dependent cell-mediated cytotoxicity mechanisms in that $F(ab')_2$ fragments of murine antibodies were used for all in vivo infusions. Secondly, in other animal studies using murine antibodies to E-selectin, similar observations were not described (25, 56, 57). Thirdly, we cannot be certain that increased vascular permeability occurred in animals treated with murine antibodies since precise kinetic and quantitative assessments of vascular leakage were not performed. It is possible that the relative degree of vascular permeability was similar in both unmodified DHR and DHR modified with mAb therapy, and edema was histologically more obvious in a context of less lymphocytic infiltration during mAb therapy. In fact, because our scoring system used the relative size of angiocentric dermal infiltrates as criterion, regardless of edema or cellular density of these infiltrates, it is possible that in animals given mAbs, the degree of inhibition of lymphocytic recruitment was underestimated. Further evaluations using computer-assisted image analysis and precise kinetic quantification of lymphocyte numbers in tissues, as well as analysis of vascular permeability from postcapillary venules, will be needed to address this issue.

With regard to the relative contribution of E-selectin versus VCAM-1 in lymphocyte recruitment in cutaneous DHR, the results of this study indicate that both proteins play significant roles, yet apparently at different periods during the evolution of the cascade. A statistically significant reduction in lymphocyte localization in skin was observed at days 2, 6, and 7 when mAb to E-selectin was administered. In contrast, when mAb to VCAM-1 was administered during DHR, a significant reduction in the number of lymphocytes was observed only at day 4. However, we cannot exclude that if more animals were examined, the statistical benefit recognized from administration of mAbs to either E-selectin, VCAM-1, or both in nonhuman primates would modify this putative temporal relationship. Nor can it be entirely excluded that antibody therapy indirectly inhibited lymphocyte recruitment by mechanisms independent from E-selectin- and VCAM-1-mediated lymphocyte endothelial adhesion. Although unlikely given the lack of inhibition seen in the animal given a similarly-prepared irrelevant antibody, further studies will be needed to address potential indirect influences of antibody administrations, such as alterations in numbers and activation of peripheral blood lymphocytes, monocyte trafficking to the inflammatory site, and systemic cytokine elaboration, on lymphocyte recruitment during cutaneous DHR or other inflammatory reactions.

The results from this study underscore the need to evaluate, on a case-by-case basis, the extent of adhesion molecule expression at inflammatory sites during experimental therapeutic trials aimed at the functional blockade of a specific adhesion molecule. Although only examined in four animals, the reduction in the numbers of lymphocytes at the inflammatory site with mAb therapy paralleled the extent of expression of the specific endothelial adhesion protein in prior DHR. Both animals receiving mAb against VCAM-1 had similar patterns of VCAM-1 expression on dermal endothelium, and both had comparable inhibition of the numbers of lymphocytes at the inflammatory site. In contrast, in the two animals receiving mAb against E-selectin, one animal had approximately twice the score for E-selectin expression than the other in DHR trials before mAb infusions, yet in the same DHR, both animals had similar numbers of lymphocytes recruited to skin. Interestingly, it was the animal with more E-selectin expression that demonstrated the greatest attenuation of lymphocyte localization at injection sites during subsequent infusions of mAb against E-selectin. Similarly, other factors that may influence outcome during blockades of E-selectin and VCAM-1 in vivo include variations in soluble concentrations of either protein in serum and the resultant effect on mAb bioavailability and concentrations of leukocyte chemoattractants at the inflammatory site. Clearly, further examination of the extent of expression of chemokines and endothelial adhesion proteins, both locally at the site and in soluble form in serum, is required to document if measurement of these mediators would be useful in determining the appropriateness of specific immunotherapy in chronic inflammatory disease.

In conclusion, our results demonstrate, for the first time, that lymphocyte recruitment in cutaneous DHR can be inhibited by the neutralization of either endothelial E-selectin or VCAM-1. Furthermore, although intuitive, our results suggest that the clinical success associated with the blockade of a specific endothelial adhesion molecule may be directly associated with the extent of expression of that endothelial adhesion molecule. Further work is required to investigate which lymphocyte subsets are most affected by E-selectin or VCAM-1 blockades, the clinical utility of simultaneous mAb therapy against both endothelial adhesion proteins, and the effect of murine mAbs directed against endothelial determinants on vascular integrity and permeability.

Acknowledgments

The authors thank Dr. Prahbat Sehgal and Lorraine Lynch for surgical assistance, Beverly Blake for editorial assistance, and Edie Estabrook for manuscript preparation.

This work was supported by awards AI25644, AI29855, and RROO168 from the National Institutes of Health.

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