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Regulation of Endothelial VCAM-1 Expression in Murine Cardiac Grafts

Expression of Allograft Endothelial VCAM-1 Can Be Manipulated with Antagonist of IFN-α or IL-4 and Is Not Required for Allograft Rejection

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This report provides evidence to support the hypothesis that tumor necrosis factor-α (TNF-α) and IL-4 promote the expression of new endothelial surface molecules in rejecting murine heterotopic cardiac allografts. The microvascular endothelia of these cardiac allografts all develop strong reactivity with the monoclonal antibodies (mAbs) YN1.1/74 (anti-ICAM-1), M/K-2 (anti-VCAM-1) and MECA-32 (undefined molecule) within 3 to 5 days of graft implantation. Daily treatment of the allograft recipients with pentoxifylline (PTX), soluble TNF receptor (TNFR:Fc), anti-interleukin-4 (IL-4) mAb (11B11), or soluble IL-4 receptor, each abrogate the expression of endothelial VCAM-1 and reduce the endothelial reactivity with the mAbs YN1.1/74 and MECA-32 to levels found in cardiac isografts. This is accompanied by a reduction, but not an elimination, of interstitial leukocytic infiltration. Despite this, cardiac allograft recipients treated with PTX or the mAb 11B11 rejected allografts at the same rate as untreated allograft recipients, ie, within 10 to 12 days after graft implantation. These rejected grafts contained mRNAs for TNF-α and IL-4, as well as for all other cytokines that have been associated with rejecting allografts. This suggests that endothelial VCAM-1 expression, which is characteristic of rejection, is not an essential element of the rejection process. Interestingly, the grafts from the PTX-treated recipients continued to display rare, isolated VCAM-1 positive cells in the interstitium, which may be dendritic cells. In general, these studies demonstrate a role for IL-4 and TNF-α in the alterations of vascular endothelial phenotype observed in rejecting cardiac allografts. They also demonstrate that endothelial VCAM-1 expression is not essential for the allograft rejection process, and that the role of VCAM-1 in this process may be more subtle than was initially suspected. (Am J Pathol 1995, 147: 166–175)

In a previous report,[1] we showed that the microvascular endothelia of murine cardiac isografts begin to express the endothelial adhesion molecule, VCAM-1, and other surface molecules associated with inflammation, within 24 hours after the graft recipients are injected with the anti-CD3 monoclonal antibody (mAb), 145–2C11. This is probably a consequence of mAb-induced polyclonal T-cell activation and subsequent cytokine production, as reported by others.[2,3] Normally, the microvascular endothelia of cardiac isografts do not develop VCAM-1 expression, although they do begin to express a new serological

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epitope that reacts with MECA-32 mAb within 3 days of graft implantation.[4] In a previous report,[5] we demonstrated that the anti-CD3 mAb-induced expression of VCAM-1 in cardiac isografts can be inhibited by simultaneous treatment of the graft recipients, either with pentoxifylline (PTX), soluble human tumor necrosis factor (TNF) receptor linked to the Fc portion of human IgG1 (TNFR:Fc), soluble IL-4 receptor, or the anti-IL-4 mAb, 11B11. This suggests that IL-4 and TNF-α are among the cytokines that can promote endothelial VCAM-1 expression in murine cardiac grafts. It further suggests that the expression of endothelial VCAM-1 that is normally observed in cardiac allografts within 3 days of implantation[4] may be a consequence of local TNF-α and/or IL-4 production. In this report, we provide data to support this hypothesis. Further, we demonstrate that endothelial VCAM-1 expression in cardiac allografts can be inhibited with a number of reagents, including PTX, and we describe the consequences for allograft rejection when endothelial VCAM-1 expression is pharmacologically eliminated from the graft.

Materials and Methods

Mice

Female DBA/2 and C57BL/6 mice 5 to 6 weeks old were purchased from Harlan Sprague Dawley (Indianapolis, IN), all pathogen-free. Mice were maintained in the Ohio State University vivarium in accordance with National Institutes of Health guidelines.

Monoclonal Antibodies

MECA 32 is a rat mAb (IgG2a) provided by Dr. Eugene Butcher (Stanford University, Stanford, CA), which was raised in rats immunized with mouse lymphoid stromal tissue. This antibody recognizes an antigen that is present on murine lymphoid tissue endothelia[6] and an inducible endothelial antigen on murine cardiac grafts.[7] The mAb does not react in immunoperoxidase analyses with murine cardiac tissues until ~3 days after the heart is transplanted into either allogeneic or syngeneic mice, at which time reactivity is confined to the graft vascular endothelia.[7] M/K-2 mAb is a rat IgG1 provided by Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK), which was generated by immunizing rats with murine bone marrow stromal cells. This antibody recognizes a murine bone marrow stromal and endothelial cell adhesion molecule, identified as VCAM-1.[8,9] COS cells that are transiently transfected with murine VCAM-1 cDNA developed reactivity with the M/K-2 mAb and adherence for very late antigen-4 (VLA-4)$^+$ Ramos cells, which are blocked by M/K-2 mAb.[9] M/K-2 mAb does not react in immunoperoxidase assays with normal cardiac tissues, cardiac isograft tissues, or cardiac allograft tissues until 3 days after transplantation into an allogeneic host, and then only with vascular endothelial cells.[4] Purified *anti-mouse* interferon-γ (IFN-γ) mAb produced in *Escherichia coli* (clone designation XMG1.2) is a rat IgG1 obtained from PharMingen (San Diego, CA). It was diluted in phosphate-buffered saline (PBS), and the mice were injected with 500 µg i.v. The mAb *11B11* is a rat anti-mouse IL-4 mAb (IgG1). The hybridoma was obtained from American Type Culture Collection (Rockville, MD), and mice were injected i.v. with ascites (produced in BALB/c nu/nu mice) containing 500 µg mAb. The mAb YN1.1/74[10,11] is a rat IgG1 that recognizes ICAM-1, obtained from the American Type Culture Collection.

Reagents

The methylxanthine derivative, pentoxifylline (3,7-dimethyl-1 (5-oxohexyl)-xanthine) (PTX) was the kind gift of Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ). PTX was diluted in saline and mice were injected with 2 µg i.v. as described elsewhere.[12] Purified, recombinant murine IL-4 was provided by Immunex Research and Development Corp. (Seattle, WA). The IL-4 was diluted in appropriate buffer containing carrier protein (bovineserum albumin; BSA) and mice were injected with 10 µg and 20 µg every 6, 8, and 12 hours i.v. Soluble murine IL-4 receptor (sIL-4R) was provided by Immunex Research and Development Corp. All Immunex protein contained less than 10 pg/µg protein. The sIL-4R was diluted in PBS, and the mice were injected with 100 µg s.c. TNFR:Fc was provided by Immunex Research and Development Corp. TNFR:Fc was diluted in PBS with a carrier protein (BSA), and the mice were injected with 100 µg s.c. Recombinant human TNF-α was obtained from Genzyme Corp. (Cambridge, MA). This reagent is effective for murine cells, and was diluted in PBS before i.p. injection into mice (100 to 500 µg/mouse).

Murine Cardiac Transplantation

Heterotopic cardiac transplantation was performed as described by Corry et al.[13] The native hearts from donor mice were harvested after heparinization of the animals and placed in iced lactated Ringer's solution while the recipient mice were prepared. The donor hearts were anastomosed to recipient abdominal

aorta and vena cava using microsurgical techniques. Upon re-establishment of blood flow the transplanted hearts resumed spontaneous contractions. Cardiac allografts undergo acute rejection with loss of function by day 10 to 12 posttransplant, whereas cardiac isografts function indefinitely.

Immunoperoxidase Staining Techniques

Various murine tissues were harvested 24 to 48 hours after treatment with anti-CD3 mAb, after cervical dislocation of the recipient. Transplanted hearts were immediately embedded in Tissue Tek ornithine carbamoyltransferase (Miles, Inc., Elkhart, IN) compound, snap frozen in supercooled isopentane, and stored at minus 80°C. Six-μm sections were fixed for 5 minutes in acetone at 4°C, and stained using standard avidin-biotin complex immunoperoxidase techniques.

All antibodies were used as undiluted tissue culture supernatants. Control sections were incubated with a 1:5 dilution of normal rat serum as the primary antibody. For endothelial staining, the secondary antibody was a biotinylated goat anti-rat IgG, which was followed by a strepavidin-horseradish peroxidase conjugate and developed in AEC, all provided in kit form (Histo-Probe anti-rat Ig staining kit, Tago, Inc., Burlingame, CA). Sections were incubated for 30 minutes with each reagent at 37°C in a humidified chamber and developed with the chromogen 3-amino-9-ethylcarbazole (AEC, 0.2% in 10% dimethyl formamide, 0.02 mol/L acetate buffer, pH 5.2, with 0.001% H_2O_2) (Sigma Chemical Co., St. Louis, MO).

RNA Extraction

Tissues were flash frozen in liquid nitrogen and stored at −80°C. For analysis, the frozen tissues were pulverized and 1.6 ml of RNA Stat-60 (Tel-Test "B", Inc., Friendswood, TX) was added. A modification of the technique of Chomczynski and Sacchi[14] was used to extract the RNA. (The solution was homogenized, then allowed to incubate at room temperature for 5 minutes. The homogenized solution was divided equally into two aliquots; each was placed over a phase lock gel (5 Prime → 3 Prime Inc., Boulder, CO). To each tube, 100 μl of chloroform (Mallinckrodt, St. Louis, MO) was added, and the mixture shaken vigorously for 15 seconds. The solution was incubated at room temperature for 3 minutes and spun at 12,000 × *g* for 15 minutes. The aqueous portion was decanted into a new microfuge tube to which 400 μl of isopropanol (Sigma Chemical Co.) was added. This mixture was vortexed and allowed to precipitate at room temperature for 10 minutes. The tubes were spun at 12,000 × *g* for 10 minutes. The supernatant was decanted. The optical density ($OD_{260/280}$) was determined after washing in 75% ethanol.

First-Strand cDNA Synthesis

RNA (5 μg) was heated to 60°C for 5 minutes and then cooled. The RNA was reverse transcribed in a 25 μl volume containing buffer (50 mmol/L Tris-Hcl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$), 3 mmol/L dithiothreitol, 50 U RNase inhibitor (USB, Cleveland, OH), 1 mmol/L (each) dNTP mix (Boehringer-Mannheim, Indianapolis, IN), 1.2 μg of oligo (dT) primer (USB), and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Grand Island, NY) for 2 hours at 37°C. Enzyme was then heat-denatured and samples were diluted to 150 μl total volume and stored at −80°C.

PCR Analysis

cDNA was analyzed in a 50 μl reaction containing 20 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs (Boehringer-Mannheim), 0.4 μmol/L primers, and 1.25 U of Taq polymerase (Perkin Elmer, Norwalk, CT). Samples were amplified under the following conditions: 94°C, 1 minute; 60°C, 2 minutes; 72°C, 1 minute for TNF-α. For IL-4, the conditions were 94°C, 1 minute; 55°C, 2 minutes; 72°C, 1 minute. All amplifications were carried out for 40 cycles. Products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Primers for IL-4 and TNF-α were generated using the computer software Oligo 4.0 (National Biosciences, Plymouth, MN) from GenBank (Genetics Computer Group, Madison, WI) sequences. Sequences are as follows: IL-4 Primer 1: 5′ ATG GGT CTC AAC CCC CAG CTA GT 3′; IL-4 Primer 2: 5′ GCI CTT TAG CCT TTC CAG CAA GTC 3′; TNF-α Primer 1: 5′ GCC CAG ACC CTC ACA CTC AG 3′; TNF-α Primer 2; 5′ AAC ACC CAT TCC CTT CAC AG 3′. All negatives were confirmed at least twice. Other cytokine primer pairs were described elsewhere.[15]

Results

In a previous study, we demonstrated that treatment of cardiac isograft recipients with PTX, sTNFR:Fc, anti-IL-4 mAb or sIL-4R interfered with the unusual expression of VCAM-1 on isograft microvascular endothelia that is caused by an i.v. injection of the graft recipient with anti-CD3 mAb.[5] The current studies were designed to determine whether these agents

could similarly interfere with the VCAM-1 expression that normally develops on cardiac allograft endothelia. To do this, DBA/2 ($H2^d$) hearts were transplanted into C57Bl/6 ($H2^b$) mice, and the graft recipients were treated daily with PTX (2 μg/day IV), with sTNFR:Fc (100 μg/day i.v.), with anti-IL-4 mAb (500 μg/day i.v.) or with sIL-4R (100 μg/day i.v.). The doses of these reagents were selected on the basis of their effects in our previous experiments.[5] Five days after allograft transplantation, the allografts were harvested and tested histologically for the expression of various endothelial surface molecules, including VCAM-1, ICAM-1, MECA-32, and were examined for evidence of cellular infiltration.

As shown in Table 1 and Figure 1, 5-day treatment of cardiac allograft recipients with PTX eliminated all graft endothelial expression of VCAM-1 (Figure 1D) that is normally observed in nontreated cardiac allografts (Figure 1A). However, PTX treatment had no obvious effects on the expression of ICAM-1 or the epitope recognized by MECA-32 mAb (Table 1). Treatment of allograft recipients with sTNFR:Fc similarly eliminated all graft endothelial VCAM-1 expression (Figure 1B); however, unlike PTX, sTNFR:Fc appeared to reduce both endothelial ICAM-1 expression and MECA-32 reactivity to the lower levels normally observed in cardiac isografts (Table 1). Like sTNFR:Fc, treatment of cardiac allograft recipients with sIL-4R eliminated all graft endothelial VCAM-1 expression (Figure 1C), and reduced endothelial MECA-32 mAb reactivity and ICAM-1 expression. So also did treatment of the allograft recipients with anti-IL-4 mAb. In addition, all four of these agents, PTX, sTNFR:Fc, sIL-4R and anti-IL-4 mAb, appeared to reduce, but did not eliminate, graft cellular infiltration.

Given the role of VCAM-1 in leukocyte adhesion[16–18] and antigen-induced T-cell activation,[19,20] we next determined whether interference with allograft endothelial VCAM-1 expression also interfered with the development of allograft rejection. Cardiac allograft recipients were treated daily with PTX (2 μg i.v. or 4 μg i.p.) or with anti-IL-4 mAb (500 μg i.v.) until the grafts lost palpable beat. At that time the allografts were removed, and the graft tissues were tested histologically for expression of endothelial VCAM-1. As shown in Figure 2, neither treatment with PTX nor with anti-IL-4 mAb prolonged the survival of the cardiac allografts, despite the complete absence of histologically detectable VCAM-1 on the graft endothelia.

The cytokines TNF-α and IL-4 have been implicated as *in vivo* inducers of VCAM-1 expression on vascular endothelial cells.[21] PTX has been shown to interfere with TNF production,[22] and with the systemic release of several additional cytokines after treatment of mice with anti-CD3 mAb.[12] To determine whether PTX interferes with the production of TNF-α or IL-4 cytokine mRNAs during rejection, cardiac allografts were obtained from PTX-treated and control mice 7 days after transplantation, and tested by reverse transcriptase-polymerase chain reaction (RT-PCR) for the presence of TNF-α and IL-4 mRNAs. The native hearts from the PTX-treated cardiac allograft recipients were used as control tissues for these experiments. All allografts were also tested by immunohistochemistry for the expression of endothelial VCAM-1. As shown in Figure 3, each of three PTX-treated grafts contained mRNAs for IL-4 and TNF-α, whereas the native hearts of the graft recipients showed little or no mRNA for these cytokines. However, none of the allografts in these studies displayed endothelial VCAM-1.

The rejected allografts from PTX-treated mice were studied further to determine what immune events occurred under conditions that disallowed endothelial VCAM-1 expression but permitted allograft rejection. Grossly, there were no obvious differences in the macroscopic appearance between PTX-treated and control cardiac allografts. Both had similar adhesions to surrounding tissues, characteristic of rejecting allografts. However, an H&E analysis of the tissues (Figure 4A) revealed that rejected hearts from PTX-treated animals had reduced tissue edema and epicardial, perivascular, and interstitial cellular infiltration as compared with nontreated, rejecting allografts (Figure 4B). In general, this resulted in an obvious reduction of tissue distortion.

As an index of local immune function in PTX-treated allografts, we also determined the profile of cytokine mRNAs that were present within the grafts when rejection was imminent. To do this, cardiac allograft recipients were treated for 10 days with PTX, the grafts were removed, and RNA was ex-

Table 1. *Evidence for Influence of TNF on Endothelial Phenotype in Murine Cardiac Allografts*

5-day graft	Treatment	VCAM-1	MECA-32	ICAM-1	INFIL
DBA/2 → DBA/2		Absent	Weak	Weak	Weak
DBA/2 → B6		Present	Present	Present	Present
DBA/2 → B6	2 μg PTX	Absent	Present	Present	Weak
DBA/2 → B6	100 μg TNFR:Fc	Absent	Weak	Weak	Weak

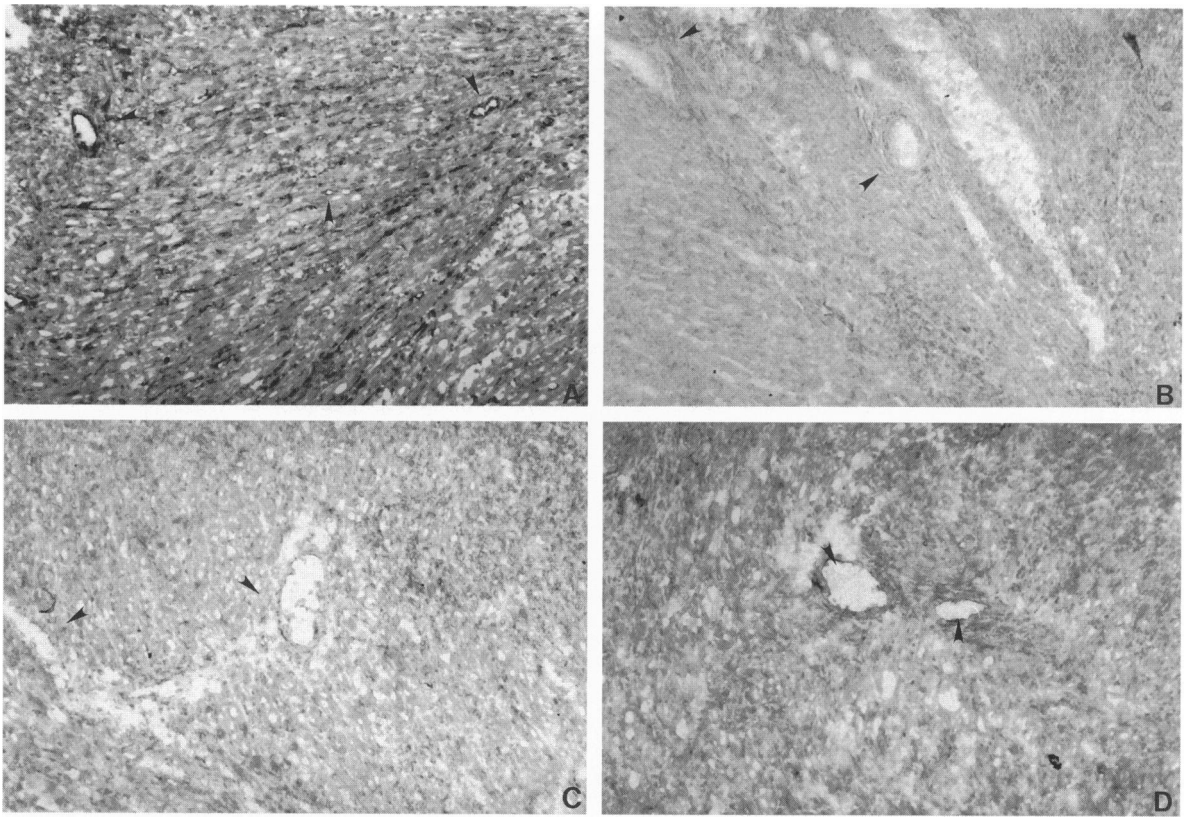

Figure 1. *Immunoperoxidase reactivity with M/K-2 mAb* (**A**) *5 day DBA/2 → C57BL/6 cardiac allograft (25×).* (**B**) *5 day DBA/2 → C57BL/6 cardiac allograft after daily treatment of the graft recipient), after treatment with sTNFR:Fc (25×).* (**C**) *5 day DBA/2 → C57BL/6 cardiac allograft after daily treatment of the cardiac allograft recipient with sIL-4R (25×).* (**D**) *5 day DBA/2 → C57BL/6 cardiac allograft after daily treatment of the allograft recipient with PTX (25×). Arrows indicate vascular structures (large vessels or microvasculature) to help with comparisons of endothelial M/K-2 reactivity.*

tracted and analyzed by RT-PCR for reactivity with various cytokine-specific primers. As shown in Figure 5, mRNA for IL-2, IL-4, IFN-γ, TNF-β, IL-1, IL-6, TNF-α, and TGF-β were all detectable in PTX-treated allografts. The same pattern of cytokine mRNAs is detectable in nontreated cardiac allografts.[15] Hence, the reduction in cellular infiltration due to PTX treatment did not eliminate the production of inflammatory cytokine mRNAs at the graft site.

Discussion

Vascular endothelial cells appear to play an active role in the process of interstitial leukocytic infiltration that characterizes allograft rejection and other chronic inflammatory responses. This process appears to be initiated by microvascular endothelial cells, which are influenced by local inflammatory mediators to display various adhesion molecules. These, in turn, interact with counter-receptors on circulating leukocytes, and promote their adhesion to vascular endothelia as the first step in their migration of leukocytes into the tissues. One endothelial adhesion molecule of interest in this regard is VCAM-1.

VCAM-1 has been identified on vascular endothelia in a wide variety of inflammatory sites, and has been shown to develop *de novo* on the vascular endothelia of rejecting human cardiac,[23–25] renal,[26,27] and liver[28] allografts. Studies with cultured human endothelial cells demonstrated that IL-1, TNF, and IL-4 are among the inflammatory mediators that can promote VCAM-1 expression in human umbilical vein endothelial cells.[29] However, IL-4 and IL-1 do not induce VCAM-1 expression in cultured human dermal endothelial cells,[30] suggesting that different endothelial beds may regulate VCAM-1 expression differently. An *in vivo* response of endothelial cells to cytokines was demonstrated by Briscoe and colleagues[21] who showed that IL-4 and TNF injected s.c. into primates acted synergistically to cause local endothelial VCAM-1 expression. Little else is known about the *in vivo* regulation of endothelial VCAM-1 expression in humans or primates.

Endothelial VCAM-1 expression also develops in a murine heterotopic cardiac allografts about 3 days

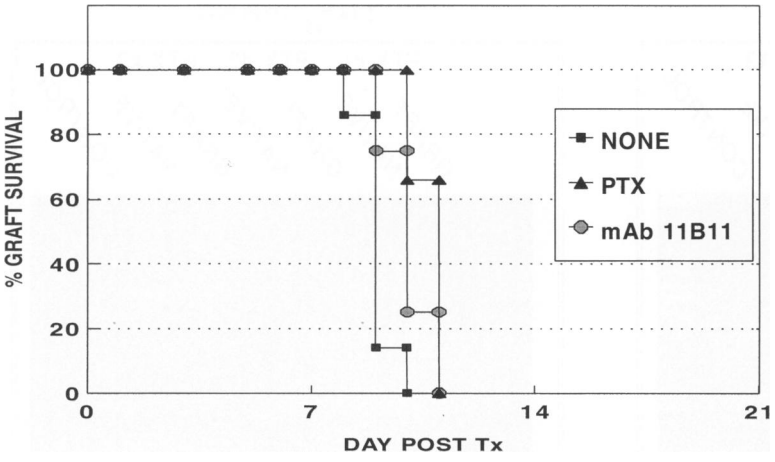

Figure 2. *Survival of cardiac allografts after treatment with PTX (△), 11B11 mAb (○), or no treatment (■). All treated grafts lacked endothelial VCAM-1 expression, as determined by immunoperoxidase reactivity with M/K-2 mAb.*

after implantation.[4] Preliminary studies in this model indicated that VCAM-1 expression on graft endothelia was dependent on the local presence within the graft of alloantigens, and of activated, graft-reactive T cells.[4] In rejecting cardiac allografts, the mRNA for IL-1 and TNF-α are detectable by RT-PCR within 24 hours of implantation, and the mRNA for IL-4 becomes detectable after 3–4 days.[15] Murine cardiac isografts, which do not develop endothelial VCAM-1 expression, also develop IL-1 and TNF-α mRNA, but no IL-4 mRNA. This suggests that IL-4 may be an important stimulus for endothelial VCAM-1 in cardiac allografts.

In the current study, we have tested this directly. We found that daily treatments of cardiac allograft recipients with either the anti-IL-4 mAb, 11B11, or with soluble IL-4 receptor could eliminate the expression of VCAM-1 by allograft vascular endothelia (Figure 1C, Table 2). This correlates with our report in a previous paper[5] that injection of cardiac isograft recipients with purified, recombinant IL-4 induced the unusual expression of VCAM-1 on cardiac isograft endothelia. These observations suggest that IL-4 is indeed part of the mechanism for endothelial VCAM-1 expression in murine cardiac allografts.

We also found, however, that treatment of cardiac allograft recipients with soluble TNF receptor could eliminate endothelial VCAM-1 expression in cardiac allografts (Figure 1B, Table 1). This suggests that TNF is also involved in the mechanism of VCAM-1 expression. Indeed, treatment of cardiac isograft recipients with purified recombinant TNF can induce VCAM-1 expression on isograft endothelia.[5] In general, our studies have led us to hypothesize that levels of endogenous TNF produced in isografts (and possibly allografts) are too low to stimulate endothelial VCAM-1 expression alone. However, when endogenous IL-4 becomes available in cardiac allografts, it synergizes with the TNF to induce endothelial VCAM-1. Hence, treatment with agents that interfere with either IL-4 or TNF also interfere with endothelial VCAM-1 expression.

Throughout these studies, we have also monitored the expression of the endothelial epitope that is recognized by MECA-32 mAb. The identity and function of the MECA-32-reactive endothelial molecule is not known, but its expression on endothelial cells has been a useful marker of endothelial behavior in the murine cardiac graft system. It differs from VCAM-1 expression in that it is displayed on both cardiac isograft and allograft endothelia, but not on endothelia of normal, nontransplanted hearts or the native hearts of the transplant recipients. In the current studies, we observed that agents that interfere with TNF-α or IL-4 availability can reduce but not eliminate the expression of endothelial MECA-32 reactivity (Tables 1 and 2). Based on our studies with murine cardiac isografts described in a previous paper[5] we hypothesize that an as-yet unidentified inflammatory stimulus is necessary to induce endothelial MECA-32 reactivity, possibly in conjunction with TNF-α. We also hypothesize that additional immune events occur in rejecting allografts, including IL-4 production, that can promote additional endothelial MECA-32 reactivity beyond levels observed in cardiac isografts. Our concurrent observations of ICAM-1 expression in cardiac allografts suggest that it is regulated similarly to MECA-32 reactivity (Tables 1 and 2), and that TNF-α and IL-4 also influence the levels of ICAM-1 expression in rejecting cardiac allografts.

The primary observations in the study involve VCAM-1 expression, which is so strikingly eliminated by the various experimental manipulations that quantitative analysis is unnecessary. Our observations regarding MECA-32 reactivity and ICAM-1 expression are meant to be generally correlative. They are sub-

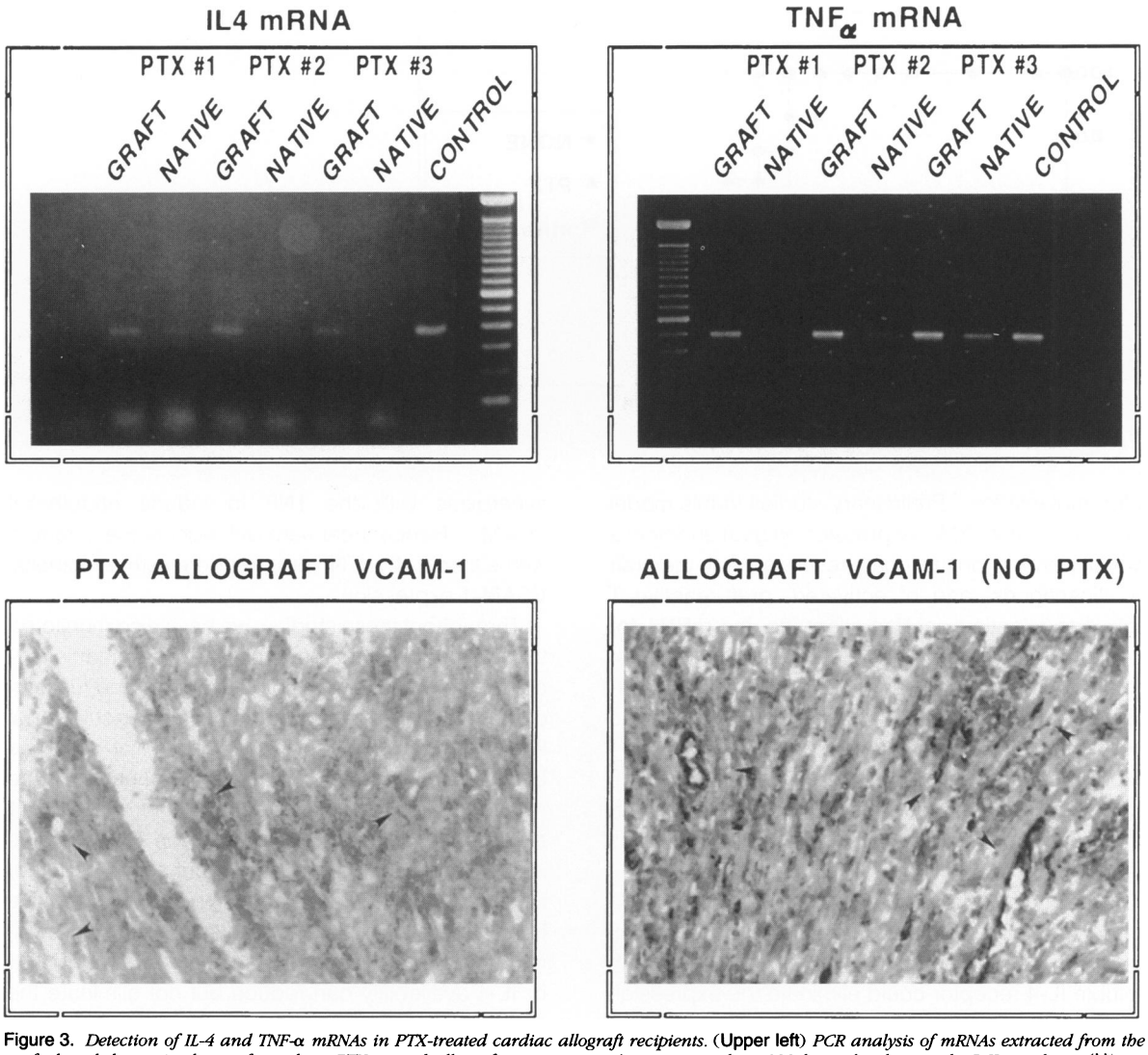

Figure 3. *Detection of IL-4 and TNF-α mRNAs in PTX-treated cardiac allograft recipients.* (**Upper left**) *PCR analysis of mRNAs extracted from the grafted and the native hearts from three PTX-treated allograft recipients. IL-4 primers produce 399 bp molecular weight PCR products.* (**Upper right**) *PCR analysis of mRNAs extracted from the grafted hearts and native hearts from three PTX-treated allograft recipients. TNF-α primers produce 500 bp molecular weight PCR products.* (**Lower left**) *Immunoperoxidase reactivity with M/K-2 mAb of cardiac allografts from PTX-treated cardiac allografts (PTX no. 1). Arrows identify vascular representative structures, which lack M/K-2 reactivity (50×).* (**Lower right**) *Immunoperoxidase reactivity with M/K-2 mAb of cardiac allografts from untreated cardiac allografts. Arrows identify vascular representative structures, with M/K-2 reactivity (50×).*

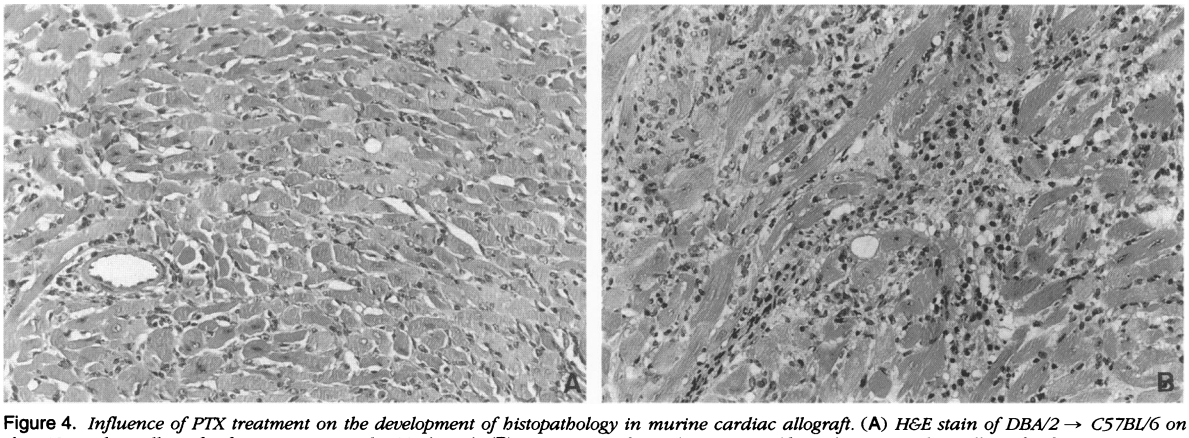

Figure 4. *Influence of PTX treatment on the development of histopathology in murine cardiac allograft.* (**A**) *H&E stain of DBA/2 → C57BL/6 on day 10 cardiac allograft after treatment with PTX (50×).* (**B**) *H&E stain of DBA/2 → C57BL/6 on day 10 cardiac allograft after no treatment (50×).*

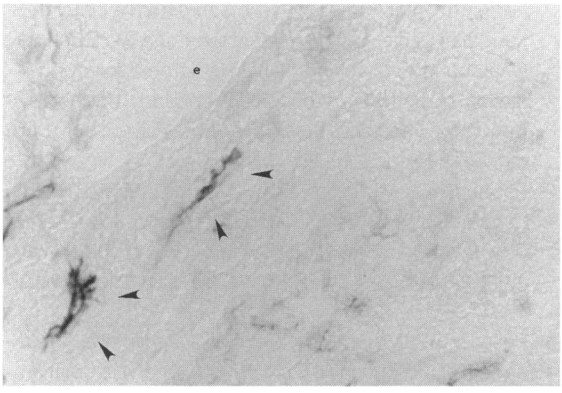

Figure 5. *Immunoperoxidase reactivity with M/K-2 mAb on day 10 in DBA/2 → C57BL/6 cardiac allograft after treatment with PTX, shown on isolated M/K-2-reactive cells present in myocardium (arrows). To facilitate visualization, the tissue was not counterstained with hematoxylin (100×).*

jective evaluations of the intensity and distribution of mAb reactivity with frozen tissue sections, and morphometric analyses of tissues remain to be done.

Another agent that interfered with endothelial VCAM-1 expression in cardiac allografts was PTX (Table 3). We initially observed that PTX could block the unusual induction of endothelial VCAM-1 expression in murine cardiac isografts that results from treatment of graft recipients with the anti-CD3 mAb, 145–2C11.[1] In this communication, we showed that PTX selectively blocks the endothelial VCAM-1 expression that normally develops in rejecting cardiac allografts (Figure 1D, Table 1). PTX is known to interfere with the production of TNF.[22] Indeed, PTX blocks the production of TNF and other cytokines that become detectable in the circulation of anti-CD3 mAb-treated mice.[12] However, we have recently observed that PTX blocks the TNF-α or IL-4-induced expression of VCAM-1 on cultured human umbilical vein endothelia (E. H. Huang, manuscript in preparation), indicating that PTX can have a direct inhibitory effect on VCAM-1 expression on endothelial cells that is independent of its inhibitory effects on the production of VCAM-1-inducing cytokines. In keeping with this, we observed that the mRNAs for IL-4, TNF-α, and TNF-β were all detectable by RT-PCR in the cardiac allografts of PTX-treated mice, despite the lack of detectable endothelial VCAM-1 (Figures 3 and 5).

Treatment of cardiac allograft recipients with PTX may have eliminated graft endothelial VCAM-1 expression, but it did not block the process of allograft rejection (Figure 2). This is not unique to PTX, since blockade of endothelial VCAM-1 expression with 11B11 mAb, the anti-IL-4 mAb, also permits the progression of allograft rejection. Treatment with these agents reduced cellular infiltration (Figure 4) and altered its composition (S. D. Bergese, unpublished observations), but did not interfere with the production of cytokine mRNAs that are normally detected in rejecting allografts, including IL-2, IL-4 and IFN-γ (Figure 5). This suggests that the mechanisms of allograft rejection do not depend on endothelial VCAM-1 expression. Studies in PTX- or 11B11-treated cardiac allograft recipients should be useful in identifying other unnecessary events that occur in rejecting murine cardiac allografts.

In previous studies, we demonstrated that the anti-VCAM-1 mAb, M/K-2, can interfere with acute allograft rejection and promote long-term (>100 days) survival of cardiac allografts in this highly stimulatory strain combination. To explain this, we initially hypothesized that the anti-VCAM-1 mAb acted by obscuring the VCAM-1 on microvascular endothelial cells. In light of the studies with PTX, we have abandoned this hypothesis. Two alternative hypotheses remain to be tested. The first is based on the observation that dendritic cells, which are probably the most antigenic of the graft cells, constituitively express VCAM-1.[27,28,31,32] We hypothesize that anti-VCAM-1 mAb interferes with local recognition of allogeneic dendritic cells at the graft site long enough for them to leave the graft.[33] This may be sufficient for the avoidance of acute rejection. In support of this hypothesis, we have observed that PTX treatment does not eliminate the expression of VCAM-1 on a very small population of isolated cells (dendritic cells?) in the allograft (Figure 5).

The second hypothesis is based on the observation that VCAM-1 is expressed within germinal centers[34] in the spleen. As an alternative hypothesis, we suggest that some critical allosensitization events associated with allograft rejection involving VCAM-1-positive cells occurs in the spleen of the

Table 2. *Evidence for Influence of IL-4 on Endothelial Phenotype in Murine Cardiac Allografts*

5-Day graft	Treatment	VCAM-1	MECA-32	ICAM-1	INFIL
DBA/2 → DBA/2		Absent	Weak	Weak	Weak
DBA/2 → B6		Present	Present	Present	Present
DBA/2 → B6	500 μg IL4 mAb	Absent	Weak	Weak	Weak
DBA/2 → B6	100 μg sIL4R	Absent	Weak	Weak	Weak

Table 3. *Allograft Cytokine mRNA Profile PTX Therapy*

Cytokine	Isograft	Allo: PTX	Allo: no Treatment
IL-1a	Present	Present	Present
IL-6	Present	Present	Present
IL-10	?	?	?
TNF-α	Present	Present	Present
TGF-β	Present	Present	Present
IL-2	Absent	Present	Present
IL-4	Absent	Weak	Weak
IFN-γ	Absent	Present	Present
TNF-β	Present	Present	Present

graft recipient. In support of this, we have observed that splenic and thymic VCAM-1 expression persists in PTX-treated allograft recipients (S. D. Bergese, unpublished observation), but a hypothesis relating this VCAM-1 expression to the process of allograft rejection is more difficult to formulate.

In summary, our studies have illustrated that endothelial VCAM-1 expression in rejecting allografts can be selectively manipulated by PTX and by a variety of agents that interfere with local TNF-α or IL-4 availability. Despite the elimination of vascular VCAM-1 by these methods, allograft rejection proceeds. This suggests that vascular VCAM-1 expression is not essential for the process of allograft rejection. Further studies in this area will identify other nonessential immune events as well as the residual events that promote graft rejection. Our studies have focused on the role of VCAM-1 in allograft rejection, which is an unusual type of antigenic insult. Nevertheless, they have revealed some unanticipated aspects of VCAM-1 regulation and function. It would be interesting to determine whether similar principles govern VCAM-1 expression and function in other VCAM-1-associated inflammatory responses, such as viral infection and autoimmunity.

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