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Transgenic Mice Expressing Different Levels of Soluble Platelet/Endothelial Cell Adhesion Molecule-IgG Display Distinct Inflammatory Phenotypes

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Platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), expressed on the surfaces of leukocytes and concentrated in the junctions between endothelial cells plays an important role in transendothelial migration of neutrophils and monocytes. Soluble recombinant PECAM-IgG injected i.v. into mice blocks acute leukocyte emigration by 80%. To study the role of PECAM in models of chronic inflammation, we generated transgenic mice constitutively expressing soluble full-length murine PECAM as an IgG chimera. Three founder lines expressed this transgene and constitutively secreted murine PECAM-IgG into the plasma where it was maintained at characteristic concentrations for each line. All mice had similar hematologic profiles to wild-type littermates and were healthy when maintained in the standard laboratory animal facility. Both the leukocytes and the endothelium of mice of all transgenic lines expressed the same levels of endogenous PECAM-1 as wild-type littermates. Similarly, there were no detectable differences in the expression of several other common leukocyte and endothelial cell adhesion molecules. Mice that produced moderate (10–20 µg/ml) concentrations of PECAM-IgG demonstrated a severely blunted acute inflammatory response, despite mobilizing appropriate numbers of circulating leukocytes. Surprisingly, mice that constitutively produced high (400–1000 µg/ml) concentrations of PECAM-IgG were unresponsive to its anti-inflammatory effects. This is the first demonstration that a soluble form of a cell adhesion molecule can be stably expressed and retain efficacy in vivo over prolonged periods. This approach is applicable to many other extracellular molecules. However, the plasma concentrations of such constitutively produced inhibitors may greatly influence the resulting phenotype. The Journal of Immunology, 1999, 163: 5640–5648.

D iapedesis, the passage of leukocytes between tightly apposed vascular endothelial cells on their way from the circulation to a site of inflammation, is a distinct step in the process of transendothelial migration (TEM). Our laboratory (1–4) and others (5–7) have demonstrated that platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) plays a unique role in diapedesis. mAbs against domain 1 and/or 2 of PECAM or soluble recombinant PECAM-IgG fusion protein containing domain 1 block TEM of neutrophils, monocytes, and NK cells in vitro and also inhibit acute inflammation in vivo (1–4, 8, 9). These reagents selectively block diapedesis of leukocytes without inhibiting the preceding adhesion steps.

The effect of these anti-PECAM reagents on chronic inflammation in vivo has not been addressed. This has not been for lack of good models, but for technical problems associated with chronic administration of the reagents. Immunocompetent animals will make Abs against foreign Igs, including the human Ig tail of PECAM-IgG. Furthermore, it is difficult to prepare sufficient quantities of these reagents in batches of similar specific activity and free of traces of contaminating endotoxin for repetitive injections over many months.

One approach to study the role of a molecule in long-term physiology or pathophysiology is to genetically delete it. However, the response of the organism to this targeted deletion is often the expression of compensatory mechanisms conducted by molecules that normally do not participate in that function or make relatively minor contributions to it, with the result that there is no significant change in phenotype.

This, in fact, appears to be the case for the recently described PECAM “knockout” mouse (10). Although anti-PECAM reagents administered to wild-type mice block leukocyte emigration by up to 80–90% (1–4, 8, 9), there is always a considerable residual leukocyte response that cannot be blocked by any concentration or combination of these agents. We demonstrated that mice with a targeted deletion in the PECAM-1 gene have a very mild phenotype, with no discernible quantitative reduction in response in several acute inflammatory models (10). We postulated that the PECAM-independent routes of transmigration, which normally account for only 10–20% of diapedesis in these inflammatory models in wild-type mice, have been expanded in the knockout mice to support the full wild-type levels of leukocyte migration.

In this report, we chose an alternative approach: to engineer transgenic mice expressing a soluble form of murine PECAM as an IgG chimera (mPECAM-IgG). This same molecule has been shown to block acute inflammation in vivo in the thiglycollate peritonitis model when administered exogenously (4). The cDNA encoding mPECAM-IgG was subcloned behind the apolipoprotein

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4 Abbreviations used in this paper: TEM, transendothelial migration; PECAM, platelet/endothelial cell adhesion molecule-1; mPECAM-IgG, fusion protein consisting of the complete extracellular region of murine PECAM fused to the Ch2 + Ch3 domains of human IgG1; bPECAM-IgG, human PECAM-IgG; PMN, polymorphonuclear cells; ApoA1, apolipoprotein A1; Tg, transgenic; CAM, cell adhesion molecule.

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A1 (ApoA1) promoter (11). Our goal was to produce mice that had circulating levels of mPECAM-IgG that would not affect normal immune surveillance or wound healing but which would be sufficient to block the enhanced leukocyte emigration that would normally ensue following an inflammatory challenge. These mice would provide us with a continuous source of genetically identical animals continuously dosing themselves with the transgene product. By using the human IgG Fc domain for these constructs, we could monitor soluble mPECAM levels and “tag” the PECAM-IgG with reagents that recognized human IgG without interfering with its ability to bind mPECAM. Because the mice begin expressing the transgene in utero, they would not recognize the human IgG as foreign. Such mice could be used to study the role of PECAM in chronic inflammatory conditions as well as to study whether they would become resistant to the anti-inflammatory effects of PECAM-IgG when administered chronically.

Materials and Methods

All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committees of The Rockefeller University and Weill Medical College (New York, NY).

Synthesis of transgenic construct

To generate soluble mPECAM-IgG chimeric protein, the extracellular domain of mPECAM was fused to the Fc region of human IgG1 (see Ref. 4 for details), and the resulting fragment was then inserted into the HindIII and XbaI sites of a plasmid vector pcDNA I (Invitrogen, San Diego, CA) in which its CMV promoter had been replaced with human ApoA1 promoter (11). The Ndel site of the CMV promoter was converted into HindIII site by linker ligation, and the PCR-generated ApoA1 promoter was inserted into this HindIII site.

Transgenic mouse production and detection

The pcDNA/mPECAM-IgG transgenic plasmid was isolated and purified by double CsCl gradient centrifugation. The purified DNA was linearized by SfiI digestion, and –5 ng of DNA was microinjected into BALB/c-fertilized oocytes from FVB/n mice, and implanted into pseudopregnant FVB/n mice at the Rockefeller University Laboratory Animal Research Center (New York, NY) in a pathogen-free environment. Genomic DNA was isolated from mouse tails biopsied after 3 wk of age, and offspring containing the integrated transgene were identified by Southern blot analysis.

Soluble chimeric protein in the sera was quantitated by ELISA using purified human IgG as standards, similar to the procedure described by Liao et al. (3). In brief, 96-well polyvinyl microtiter plates were coated with 25 μg/ml of purified goat anti-human Fc Ab (Pierce, Rockford, IL), nonspecific binding was blocked with PBS containing 0.1% OVA, and dilutions of the test sera were then incubated on the treated plates, which were then washed extensively. Bound chimera was detected with alkaline phosphatase-conjugated goat anti-human Fc polyclonal Ab (Pierce) and substrate (p-nitrophenyl phosphate) in Aurophos substrate buffer (JBL Scientific, San Luis Obispo, CA). Fluorescence was quantitated on a Cytofluor 3500 (PerSeptive Biosystems, Framingham, MA) (3).

The molecular size of the transgenic protein was determined by Western blot: 10 μl of sera was fractionated on 4–16% SDS-PAGE. Proteins were immobilized onto polyvinylidene difluoride membrane (Millipore, Bedford, MA) and probed with HRP-conjugated goat anti-human IgG Ab. Transgene-positive mice were then crossed to FVB/n mice to establish founder lines, from which heterozygous mice were intercrossed to obtain mice homozygous for the transgene.

In an additional approach, tissues were homogenized in a electric tissue homogenizer (Omni International, Waterbury, CT) in 0.1% Nonidet P-40 (Sigma, St. Louis, MO) and protease inhibitors. Equal amounts of protein, as determined by BCA protein assay (Pierce), were separated by SDS-PAGE and subjected to immunoblot as above.

Hematological parameters

All of the mice, from three separate founder lines, were weighed, and peripheral bloods were taken from age- and sex-matched transgenic-positive and -negative littermates. Complete white blood cell counts and differential counts were measured by the Diagnostic Laboratory of the Weill Medical College Research Animal Resource Center.

Quantitative PCR

A variety of tissues were excised from age-matched transgenic and nontransgenic mice. Approximately 100 μg of fresh tissue was immersed in 1 ml of Trizol solution (Life Technologies, Gaithersburg, MD) and total RNA was isolated and purified according to the manufacturer’s standard protocol. Contaminating DNA in the resulting RNA samples was eliminated by digestion with RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN). RT-PCR was performed by using the primers listed below and TaqMan probes (Perkin-Elmer, Foster City, CA) using previously published methods (12, 13). The copy number of RNA message of both transgenic and endogenous PECAM genes were quantitated by using plasmids containing the transgenic construct and murine full-length PECAM cDNA as standards. Values are expressed as copies of mRNA per 50 ng of total RNA, normalized with house-keeping gene, GAPDH.

The nucleotide sequences of primers and TaqMan probe are as follows: Endogenous mPECAM cytoplasmic domain forward primer, 5′-CT GAACTTCAACAGCGGAGAAGCT-3′; reverse primer, 5′-TCAAGGAGAGGCACTTCCACTTT-3′; and TaqMan probe, 5′-TGTTAGAAGCACAACGCCATTACCGTT-3′. Human IgG1 Fc: forward primer, 5′-GTGAGGACGAGGACCTGTA-3′; reverse primer, 5′-GACCTTGCTGATCTGACTCTGCC-3′; and TaqMan probe, 5′-ACACCACTACCTACCAATGTCGCC-3′. Murine GAPDH: forward primer, 5′-GACTCCTTCTTGGAGGTGAGCTCC-3′; reverse primer, 5′-TTCGGCTAGGTGGAGCTACTAC-3′; and TaqMan probe, 5′-TTCGAGTGGCAATTGGAGTGTGTC-3′.

Flow cytometry

Approximately 25 μl of heparinized mouse blood was diluted in 100 μl of PBS containing 20 mM EDTA and 0.1% human serum albumin. RBCs were lysed by adding 2 ml of lysis buffer (1.66% ammonium chloride) and incubating for 10 min at room temperature. After a single centrifugation (1200 rpm for 5 min) the white blood cells were resuspended in cold HBSS. Single cell suspensions were incubated for 30 min at room temperature with primary Abs, washed twice as above, and incubated for an additional 30 min with FITC-conjugated secondary Abs, if necessary. Analysis was performed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA).

For some experiments using mPECAM-IgG, fibroblasts transfected with pMCEK were subjected to flow cytometry and analyzed as in Ref. 4.

Histochemistry

Organs from freshly killed mice were surrounded with OCT (Miles Laboratories, Elkhard, IN) and snap-frozen by immersion in liquid nitrogen. Frozen sections were cut 5 μm thick, picked up on gelatin-coated glass slides, fixed in acetone for 5 min before storage at –20°C. Immunoperoxidase histochemistry was performed as described (2) by using rat anti-mouse PECAM-1, Vcam-1, ICAM-1, and ICAM-2 (all from Pharmingen, San Diego, CA) and peroxidase-labeled rabbit anti-rat IgG (Dako, Carpintera, CA).

To detect endogenous mPECAM in tissues in the presence of a vast excess of soluble mPECAM-IgG, immunohistochemistry was performed using a rabbit antiserum generated against the cytoplasmic tail of mPECAM (generously provided by Dr. André Veillette, McGill Cancer Center, Montreal, Canada) diluted 1:500 and detected with peroxidase-labeled swine anti-rabbit IgG (Dako). Reaction product was developed with diaminobenzidine-H2O2. Slides were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted in Permount (Fisher Chemical, Fairlawn, NJ).

Thioglycollate broth-induced peritonitis

These studies were performed and analyzed as previously described (2). Measurements (animal weight; peritoneal lavage volume, cell density, and differential count; peripheral blood count and differential; and general autopsy) were generally performed 18 h after i.p. injection of 1 ml of 4% thioglycollate broth. In each experiment, age- and sex-matched littermates were used as controls. Over the course of these studies, mice ranging in age from 8 wk to 6 mo were used in individual experiments. There was no difference in the inflammatory response of any strain as a function of age within the range tested.

Isolation and purification of mPECAM-IgG from transgenic sera and serum transfer experiment

The transgenic protein was purified from 10 ml of serum drawn from Tg11 mice by affinity chromatography using CNBr-activated Sepharose coupled
to rabbit anti-human Fc Abs (Pierce), preabsorbed with mouse serum. The bound protein was eluted with 0.1 M glycine (pH 2.5) and neutralized with 1/10 vol of 1 M Tris-HCl. After dialysis, the pure protein was dissolved in PBS and filter-sterilized before injecting animals.

 Appropriately diluted serum from Tg11 mice (10-fold or 4-fold diluted) or comparable quantities of purified transgenic protein injected i.v. into wild-type mice several hours before thioglycollate injection i.p. The inflammatory response of the recipient mice was analyzed 18 h later. Serum from age-matched wild-type mice was used as a control.

**PECAM-IgG dose response**

To determine whether PECAM-IgG lost its ability to block TEM at high concentrations, assays were conducted in vitro using human PECAM-IgG (hPECAM-IgG), HUVEC, and human monocytes. These assays were performed as previously described (4) except that the concentrations of PECAM-IgG ranged from 1 μg/ml up to 1 mg/ml.

**Statistics**

Nonparametric data were evaluated by the Mann-Whitney U test using JMP software (SAS, Research Triangle Park, NC).

**Results**

**Characterization of mPECAM-IgG transgenic mice**

Three founder lines were generated by using the transgene construct containing the ApoA1 promoter (Fig. 1a). Mice of all three lines were the same size and weight as their wild-type littersmates. They were healthy and fertile and had normal ratios of male and female offspring. The mice were maintained in a clean (but not specific pathogen-free) environment, had normal life spans, and had no apparent increase in susceptibility to infectious diseases from environmental organisms or tendency for wounds and lacerations to become infected (data not shown).

Quantitative PCR demonstrated that the transgene was expressed in the liver and lung, with lower levels in the kidney. The Tg11 strain showed expression in splenic tissue, which was not detected in the lower secreting Tg8 mice. (Table I). The mice showed no spontaneous bleeding tendencies, and their hematologic profiles were similar to their wild-type littersmates (Table II).

The mice secreted intact mPECAM-IgG into the circulation. Mice of the Tg8 (moderate producer) line heterozygous for the transgene had plasma levels of ~10 μg/ml as determined by ELISA; homozygotes had levels of ~20 μg/ml. Mice of the Tg5 and Tg11 lines (high producers) heterozygous for the transgene generally expressed between 500 and 800 μg/ml. Lower levels (100–400 μg/ml) were produced with advanced age. Homozygous mice of these lines produced approximately double these levels, but the phenotype observed was the same as for the heterozygotes (see below). The higher levels of circulating PECAM-IgG in Tg11 correlated with increased message levels for the transgene, especially in the liver and lung (Table I) but not with intensity of the hybridization signal on Southern blot (data not shown). Immuno-blot of transgenic serum demonstrated that the PECAM-IgG was running as a single band of 230 kDa, the expected size of the intact soluble form of mPECAM (open box) fused to the Fc portion of human IgG1 (hIgG, hatched box) in the pcDNA1 vector (4). The solid black bar to the left of domain 1 represents the nPECAM signal sequence; numbers 1–6 refer to the Ig domains of PECAM; letters below the construct indicate restriction enzyme sites: H, HindIII; N, NolI; X, Xbal. b. The expression and the molecular size of the mPECAM-IgG transgenic protein was detected by Western blot analysis. Serum (10 μl) from the Tg11 transgenic mouse (TG) and its wild-type littermate (WT) were fractionated on the 4–16% SDS gel under the nonreducing conditions, and the resulting membrane was probed with goat anti-human IgG Ab. c. PECAM-IgG was purified from serum of Tg11 mice using protein A-Sepharose. Five micrograms was subjected to SDS-PAGE under nonreducing (NR) or reducing (R) conditions and stained with Coomassie brilliant blue. Positions of m.w. standards run on an interposing lane (S). The double-headed arrow indicates the position of intact mPECAM-IgG in b and c. The single arrow to the right of lane (R) indicates the position of the reduced form of mPECAM-IgG. d. Murine lung from wild-type and transgenic lines (line number indicated above the lanes) was homogenized, and aliquots containing equal total protein were subjected to SDS-PAGE under nonreducing conditions. Western blot probing for PECAM-IgG in the tissue plasma and interstitial fluid demonstrate none in wild type, whereas abundant PECAM-IgG (band at 230 kDa) is detected in homogenate of Tg5 and Tg11 tissue. Numbers on the sides of b and d indicate positions of molecular mass markers (in kDa).

**Table I. Expression of transgene mRNA**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lung</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.7</td>
<td>3.4</td>
<td>ND</td>
<td>10.0</td>
</tr>
<tr>
<td>Tg8</td>
<td>8,700</td>
<td>ND</td>
<td>1,700</td>
<td>4,600</td>
</tr>
<tr>
<td>Tg11</td>
<td>920,000</td>
<td>1,900,000</td>
<td>930</td>
<td>4,000,000</td>
</tr>
</tbody>
</table>

a The figures represent the copies of transgene-specific mRNA per 50 μg total RNA as determined by quantitative Taq-Man PCR. The duplicate numbers represent the duplicate samples run. ND = not detected (<1 copy/50 μg).
compared to wild type and Tg8 or Tg11, no differences were found in any category.

Expression of PECAM and CD11/CD18.

Control experiments probing the same samples with an Ab against human IgG identified only PECAM-IgG in the samples from transgenic mice (Fig. 1d). Note the absence of a band at 135 kDa, the size of endogenous PECAM, in these samples (Fig. 1d). The same Ab was used in immunohistochemical studies on frozen sections of kidneys from these mice. The staining pattern of all vascular structures was identical in the wild-type mice and all three transgenic lines. Even the relative intensity of staining (arterioles > venules > glomeruli > peritubular capillaries) was the same in all lines (data not shown).

No difference in staining pattern or intensity was detected between wild-type and any transgenic line when probed using Abs against ICAM-1, ICAM-2, or VCAM-1. In the kidney specifically, ICAM-2 was expressed by endothelial cells of all vessels, including glomerular capillaries (data not shown). ICAM-1 staining in kidneys was weak; and VCAM-1 staining was focally and weakly present on endothelium of some of the larger microvessels but was absent from peritubular and glomerular capillaries (data not shown). Thus, except for the presence of circulating PECAM-IgG, the transgenic mice do not appear different from their wild-type littermates under basal conditions.

**Response to inflammatory challenge**

Wild-type FVB/n mice and all strains of transgenic mice had similar numbers of mononuclear cells and virtually no polymorphonuclear cells (PMN) resident in their peritoneal cavities when unstimulated (Figs. 5 and 6). When wild-type FVB/n mice were challenged with an i.p. injection of thioglycollate broth and sacrificed 18 h later, a dramatic inflammatory exudate including large numbers of neutrophils as well as monocytes was seen (Fig. 5). In contrast, age-matched Tg8 (moderate producer) littermates showed a marked blunting of the inflammatory response. Heterozygous mice that have circulating mPECAM-IgG levels of 10 µg/ml showed a 50–70% reduction in PMN numbers; monocytes were

**FIGURE 2.** Expression of PECAM and CD11/CD18 family members is not altered on transgenic mouse leukocytes. After lysis of RBC and extensive washing to remove traces of plasma, leukocytes from transgenic and wild-type mice were incubated with mAb against either PECAM, CD11a (rat anti-mouse), CD18 (hamster anti-mouse), or CD62L (rat anti-mouse). Appropriate FITC-conjugated secondary Abs were applied after several washes. PBMC and PMN were gated separately based on established forward scatter/size scatter properties. The profiles shown are from a single experiment representative of at least four. Dark profiles represent staining with the specific anti-PECAM mAb. Open profiles represent the position of isotype-matched negative control Ab.

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<table>
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<tr>
<th>Strain</th>
<th>RBC (×10^12/ml)</th>
<th>Platelets (×10^12/ml)</th>
<th>WBC (×10^12/ml)</th>
<th>PMN (%)</th>
<th>Lymph (%)</th>
<th>Mono (%)</th>
<th>Eos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>7.46 ± 0.42</td>
<td>1.13 ± 0.10</td>
<td>7.89 ± 0.48</td>
<td>16.4 ± 0.8</td>
<td>82.6 ± 2.4</td>
<td>0.9 ± 1.1</td>
<td>0.1 ± 0.4</td>
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<tr>
<td>Tg5</td>
<td>8.17 ± 0.22</td>
<td>1.34 ± 0.05</td>
<td>11.5 ± 0.8</td>
<td>19.4 ± 1.5</td>
<td>79.0 ± 5.1</td>
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<td>0.4 ± 0.7</td>
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<tr>
<td>Tg8</td>
<td>7.68 ± 0.36</td>
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<td>8.37 ± 0.81</td>
<td>24.4 ± 13.8</td>
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</tr>
<tr>
<td>Tg11</td>
<td>8.48 ± 0.08</td>
<td>1.33 ± 0.13</td>
<td>10.8 ± 0.94</td>
<td>17.1 ± 2.5</td>
<td>83.7 ± 6.7</td>
<td>0.3 ± 0.7</td>
<td>0.1 ± 0.4</td>
</tr>
</tbody>
</table>

* The data shown are from one experiment in which all four strains were tested simultaneously. In several other experiments, performed at different times, in which Tg5 was compared to wild type and Tg8 or Tg11, no differences were found in any category.

* Different from WBC counts of other groups at p ≤ 0.05. There is no significant difference among strains in any other category at p = 0.05.

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The Journal of Immunology
FIGURE 3. a–d, Mouse leukocytes do not carry mPECAM-IgG. Mouse blood cells were stained with FITC-labeled goat anti-human IgG to detect the presence of bound mPECAM-IgG molecules and processed for flow cytometric analysis as in Fig. 2. No mPECAM-IgG is detected on Tg11 (a) or wild-type (c) leukocytes even though it circulated at high concentration (1 mg/ml) in Tg11, as detected by ELISA. As a control, aggregated normal human IgG did bind to both Tg11 (b) and wild-type (d) leukocytes when it was added at the same concentration, presumably bound via low affinity Fc receptor. e–h, mPECAM-IgG does not bind to high affinity Fc receptors. Leukocytes from wild-type (e), Tg8 (f), Tg5 (g), and Tg11 (h) mice were extensively washed free of plasma and incubated for 30 min with (thick line) or without (thin line) purified PECAM-IgG from the serum of Tg11 mice at 1 mg/ml. Cells were then washed again and subject to analysis by flow cytometry after staining with FITC-labeled goat anti-human IgG. a–d, Histograms gated on all leukocytes; e–h, histograms gated on neutrophils. i–l, mPECAM-IgG binds homophilically to mouse PECAM. Fibroblasts transfected with mPECAM or control vector were incubated with mPECAM-IgG (20 µg/ml) purified from Tg11 serum. After washing, bound PECAM-IgG was detected with a FITC-labeled anti-human Fc Ab (4). mPECAM-IgG binds to transfectants bearing mPECAM (thick line in i) but not to control transfectants (thick line in k). mAb 2H8 against mPECAM also bound to transfected cells (j) but not to controls (l). No staining was seen in the absence of primary reagents (thin lines in i–l).

Reduced to near basal levels (Fig. 5 and Table IV). In homozygous mice producing 20 µg/ml, PMN infiltration was blocked by >80% (Fig. 5 and Table IV). This level of plasma mPECAM-IgG was similar to the levels that were achieved in experiments in which exogenously administered mPECAM-IgG was found to block PMN emigration into the peritoneal cavities of wild-type mice by 80% (4). Circulating leukocyte counts in Tg8 mice receiving thioglycollate broth were elevated at 18 h, as had been seen previously with wild-type mice receiving anti-PECAM reagents in the face of a thioglycollate challenge (2, 4) consistent with the notion that leukocytes could be mobilized normally but could not enter the site of inflammation.

Table III. Expression of endogenous mPECAM-1*  

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lung</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
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<tr>
<td>Wild type</td>
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<td>2.8 x 10^3</td>
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<td>Tg8</td>
<td>8.4 x 10^3</td>
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<tr>
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<td>9.8 x 10^3</td>
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<td>8.4 x 10^4</td>
<td>6.7 x 10^3</td>
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<td>5.9 x 10^2</td>
<td></td>
</tr>
</tbody>
</table>

The figures represent the copies of mRNA encoding endogenous mPECAM per 50 µg total RNA as determined by quantitative Taq-Man PCR. The duplicate numbers represent the duplicate samples run. The sample for Tg8 spleen was lost in this experiment.

The block in inflammation was at the level of TEM, as has been found previously (2, 4). Histologic sections of tissues in the Tg8 mice showed PMN and monocytes concentrated intravascularly in postcapillary venules, many apparently attached to the endothelial surface, but unable to transmigrate (data not shown) as occurs with anti-mPECAM mAb (2) or exogenously administered mPECAM-IgG (4). Table IV summarizes the results obtained in all of the experiments performed over the course of this study.

Too much of a good thing

A surprising result came when evaluating the high producer lines of transgenic mice. The response to i.p. thioglycollate was virtually the same as for wild-type mice at both 3 and 6 h (data not shown) and at 18 h (Fig. 6). Peritonitis in response to thioglycollate broth was blocked, as expected, in the high producer strains by Abs against CD11a and CD11b (Fig. 6). This result indicates that these mice were using leukocyte β2 integrins for adhering to the vascular wall.

The failure of the transgenic protein to block leukocyte migration in the high producer mice was not due to defects in the transgenic protein itself: serum (Fig. 7a) or purified transgenic protein (Fig. 7b) from these mice transferred to wild-type mice blocked

FIGURE 4. Transgenic mice express wild-type (WT) levels of endogenous PECAM. Aliquots of lung homogenates containing equal total protein were subjected in duplicate to immunoblot analysis as in Fig. 1d. The polyvinylidene difluoride membrane was probed with a rabbit antisem generated against the cytoplasmic tail of mPECAM (17) and developed with HRP-labeled swine anti-rabbit IgG. The arrow indicates the band that develops runs at 135 kDa, the expected size of endogenous mPECAM. Within the limits of sensitivity and reproducibility of the assay, no difference in the expression of endogenous PECAM was detected.

FIGURE 5. Inflammatory response is blunted in Tg8 mice. Mice were sacrificed 18 h after receiving thioglycollate (Thio) broth i.p. Infiltration of both PMN and macrophages both showed a dramatically blunted response compared with their wild-type littermates. Homozygous mice (Tg8++), which produced twice the amount transgenic protein as heterozygotes (Tg8+/−), blocked leukocyte migration by >80%. The results shown are the mean ± SEM from five mice/group. The data are from one experiment representative of five. All values for PMN are significantly different from WT + Thio (p = 0.001). For monocytes (Mo), Tg8 heterozygotes significantly different from WT + Thio (p = 0.05); Tg8 homozygotes significantly different from WT + Thio (p = 0.01).
inflammation well and in a dose-dependent fashion. Fig. 7 shows data using mPECAM-IgG from the Tg11 strain; similar results were obtained using sera from the Tg5 strain (data not shown).

Many chemoattractants, chemokines, and Abs whose actions rely on cross-linking their ligands exhibit a bell-shaped dose-response curve. If the blocking effect of PECAM-IgG exhibited such properties, it was possible that the high levels of mPECAM-IgG in the plasma of Tg5 and Tg11 mice were well beyond the optimal inhibitory point and were, in fact, no longer effective. Alternatively, if PECAM-IgG bound to itself at high concentrations producing aggregates in which the binding sites of PECAM were already occupied, a decrease in efficacy as concentration rose above a certain threshold might be expected.

We initially tried to test these hypotheses in vivo by infusing mice i.v. with 200 µl of mPECAM-IgG from Tg11 mice at a concentration of 6 mg/ml (the highest concentration we could achieve without running into viscosity problems) 1 h before administration of thioglycollate broth. The serum half-life of mPECAM-IgG at 10 – 20 µg/ml was maintained up to the highest dose. Therefore, it is unlikely that the high levels of mPECAM-IgG in the plasma of Tg5 and Tg11 mice were well beyond the optimal inhibitory point and were, in fact, no longer effective. Alternatively, if PECAM-IgG bound to itself at high concentrations producing aggregates in which the binding sites of PECAM were already occupied, a decrease in efficacy as concentration rose above a certain threshold might be expected.

We initially tried to test these hypotheses in vivo by infusing mice i.v. with 200 µl of mPECAM-IgG from Tg11 mice at a concentration of 6 mg/ml (the highest concentration we could achieve without running into viscosity problems) 1 h before administration of thioglycollate broth. The serum half-life of mPECAM-IgG is ~24 h. With this dose, we were able to achieve a serum concentration that was still ~250 µg/ml at the time of sacrifice. This concentration blocked influx of leukocytes as well as the lower concentrations did (data not shown). Because we could not reasonably achieve higher concentrations of PECAM-IgG, we decided to investigate this question in a system that we could better manipulate. Thus, experiments were conducted in vitro using human leukocytes and endothelium and using hPECAM-IgG in an assay that we had previously demonstrated to rely on homophilic PECAM-PECAM interactions in the same manner as the in vivo peritonitis assay (Fig. 8).

In previous studies, we had focused on how little PECAM-IgG was necessary to block monocyte transmigration. In dose-response experiments, optimal blockade was seen at 50 nM (4), but the experiments were seldom conducted above 100 µM (20 µg/ml). To see whether there was a biphasic dose-response to PECAM-IgG in vitro, we repeated these experiments exposing monocytes to concentrations of up to 1 mg/ml PECAM-IgG. The results, shown in Fig. 8, demonstrate that the plateau blockade by PECAM-IgG is maintained up to the highest dose. Therefore, it is unlikely that the failure of Tg5 and Tg11 leukocytes to be blocked by the high concentrations of PECAM-IgG circulating in their plasma is due to a decreased response to these concentrations.

**Discussion**

Neutrophils and monocytes from transgenic mice expressing mPECAM-IgG at 10–20 µg/ml were blocked at the level of TEM from entering a site of acute inflammation. To our knowledge, this is the first successful attempt at using a transgenic cell adhesion
molecule (CAM)-IgG to inhibit inflammation in vivo. Thus, blocking PECAM with therapeutic concentrations of this circulating inhibitor will block leukocyte emigration, and the mice will not find a way around it, even though they have been exposed to it at these concentrations beginning in utero and continuing into their adult life.

These mice are presently being mated to introduce the PECAM-IgG transgene into strains that are susceptible to atherosclerosis (ApoE-deficient), collagen-induced arthritis (DBA/1), and experimental allergic encephalitis (SJL) to determine whether chronic exposure to therapeutic levels of circulating PECAM-IgG will effectively inhibit leukocyte emigration, and hence disease, in those models.

Because two lines of mice produced from this same transgene construct did not have a noticeable defect in acute inflammation, one might wonder whether the block of acute inflammation seen in the Tg8 mice producing 10–20 mg/ml of mPECAM-IgG was unique and nonreproducible. In fact, however, we have produced an independent line of transgenic mice expressing mPECAM-IgG behind a different promoter. These mice constitutively express mPECAM-IgG at 25 mg/ml and display a phenotype similar to that of Tg8. That is, they are healthy and have normal levels of circulating leukocytes but have a severely blunted response to i.p. injection of thioglycollate broth (data not shown).

Our data strongly suggest that it is the level of circulating mPECAM-IgG to which the transgenic animals are chronically exposed that determines the phenotype. When the plasma levels are below a certain threshold, transmigration can proceed at a basal level that is sufficient for normal wound repair and homeostasis in a pathogen-free environment. However, the levels of mPECAM-IgG in these mice is sufficient to block transmigration in an inflammatory response when the mice are acutely challenged. On the other hand, chronic exposure to supratherapeutic levels (~400 μg/ml) of mPECAM-IgG apparently lead to unresponsiveness to its anti-inflammatory effects. Exposure of the transgenic mice began in utero; however, it is possible that chronic exposure to such high levels of mPECAM-IgG suppresses leukocyte emigration.

**FIGURE 7.** Transgenic mice expressing high concentrations of mPECAM-IgG make a functional product. Wild-type mice received 100 μl of normal mouse serum (NMS) or 100 μl of serum from Tg11 mice diluted into NMS 1 h before i.p. injection of thioglycollate (Thio) broth. Mice were sacrificed 18 h later and peritoneal inflammation quantitated as in previous figures. *a*, Serum from Tg11 mice blocked the inflammatory response in wild-type mice when administered exogenously. Diluted serum (4-fold) blocked more efficiently than 10-fold dilution. *b*, The transgenic protein, when purified from the Tg11 serum and injected into wild-type mice at a concentration adjusted to match the original Tg11 serum (pu. Tg Protein), blocked inflammation as well as the Tg11 serum from which it was purified. Data show mean ± SEM for five mice/group and are representative of three independent experiments. All groups are significantly different from NMS + Thio. For PMN, *p* = 0.001; for monocytes (Mo), *p* = 0.05. Tg11 serum and purified transgenic protein are not significantly different from each other.

**FIGURE 8.** Dose response of hPECAM-IgG in blocking monocyte transmigration in vitro. Monocyte transmigration across the HUVEC monolayer grown on collagen gel was performed as described previously (1, 3). Soluble recombinant hPECAM-IgG was added to culture medium at different doses (in μg/ml) as indicated in the figure to block monocyte migration. Control monolayers received medium only. As a positive control, mAb hec7 was added to groups of monolayers at optimal doses to block TEM. All groups are significantly different from control at *p* = 0.001; hec7 and all doses of hPECAM-IgG ≥ 10 μg/ml were not significantly different from each other at *p* = 0.001.
levels of anti-PECAM reagents beginning later in life could produce similar effects. This result would have important implications for dosing anti-PECAM therapy.

Those transgenic mice constitutively expressing high levels of PECAM-IgG were apparently resistant to its effects. The mechanism for this resistance is not clear but was not due to 1) down-regulation of PECAM-1 on either leukocytes or endothelial cells, 2) binding of mPECAM-IgG to leukocyte Fc receptors, 3) production of an ineffective transgene product, or 4) intrinsic ineffectiveness of PECAM-IgG inhibition at high doses. At high concentrations, PECAM will bind to itself homophilically (14). However, we do not think that homophilic aggregation, rendering PECAM aggregated by targeted deletion.

Proper function of PECAM requires that the cells are desensitized to PECAM at the signal transduction level, how long this desensitization lasts if they are removed from the high-dose mPECAM-IgG environment of the mouse, or whether they simply override or bypass the block in PECAM function by using alternative molecular pathways. The transgenic mice expressing moderate levels of mPECAM-IgG will be useful in testing the efficacy of long-term anti-PECAM therapy in models of chronic inflammation. The transgenic mice expressing high levels of mPECAM-IgG will be useful tools for investigating the PECAM-independent pathways of TEM and, perhaps, PECAM signaling. This technique has broader implications, however, because it can be applied to other adhesion molecules and other extracellular proteins that can be inhibited by soluble decoys. The anti-inflammatory reagents are not limiting and (due to constitutive production beginning in embryogenesis) nonimmunogenic. Thus, similar transgenic mice could be made to study the roles of other (adhesion) molecules in large populations of genetically identical mice over periods as long as the life span of the animal.

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