Involvement of Platelet–Endothelial Cell Adhesion Molecule–1 in Neutrophil Recruitment in Vivo

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During inflammation, neutrophils migrate from the vascular lumen into extravascular sites. In vitro assays have suggested that platelet–endothelial cell adhesion molecule–1 [PECAM-1 (CD31)], a member of the immunoglobulin superfamily, is required for the transmigration of neutrophils across endothelial monolayers. Antibody to human PECAM-1, which cross-reacts with rat PECAM-1, was found to block not only in vivo accumulation of rat neutrophils into the peritoneal cavity and the alveolar compartment of the lung but also neutrophil accumulation in human skin grafts transplanted onto immunodeficient mice. On the basis of these findings in three different models of inflammation, it appears that PECAM-1 is required for neutrophil transmigration in vivo and may thus be a potential therapeutic target.

The PECAM-1 adhesion molecule, also known as CD31 or endoCAM, is expressed in large amounts on endothelial cells at intercellular junctions and to a lesser extent on platelets and most leukocytes (*1–5*). It is structurally related to other cell-cell adhesion molecules [such as ICAM-1, VCAM-1, and N-CAM (*1*)], is localized at surface borders of adjacent endothelial cells and fibroblasts transfected with PECAM-1 complementary DNA (cDNA) (*2*), and can mediate aggregation of L cells transfected with PECAM-1 cDNA (*2, 6*). In vitro studies have implicated PECAM-1 in the initiation of endothelial cell contact (*7*), capillary tube formation (*8*), and neutrophil and monocyte transmigration through an endothelial cell monolayer (*9*). The function of PECAM-1 in an intact animal model has not yet been determined.

Because bioactive monoclonal antibodies to nonhuman forms of PECAM-1 are unavailable, we took advantage of the cross-reaction of a rabbit polyclonal antibody generated by immunization with purified human PECAM-1 (*2*) with rat PECAM-1 (*8*). The specificity of the antibody (anti–PECAM-1) was established by showing that the antibody (i) stains rat endothelial cell-cell junctions in culture and immunoprecipitates a 130-kD protein from microvascular endothelial cells from rat epididymal fat pads (*8*), (ii) immunostains a panel of rat tissues, which demonstrates strong constitutive reactivity only with endothelial cells (Fig. 1), and (iii) reacts only weakly with the surface of neutrophils and lymphocytes by flow cytofluorometry analysis. This antibody [as well as its Fab and $F(ab')_2$ fragments] is bioactive: It inhibits the aggregation in vitro of L cells transfected with the cDNA for human PECAM-1 (*2*) and blocks the formation of rat endothelial capillary tubes (*8*).

We injected anti–PECAM-1 into rats to assess if it would alter the neutrophil accumulation induced by either glycogen instilled into the peritoneal cavity (*10*) or the deposition of immunoglobulin G (IgG) immune complexes in the lung (*11*). Both models require the participation of E-selectin, ICAM-1, and CD11-CD18 integrins (*10, 12*). Glycogen in the rat peritoneal cavity caused accumulation of $39.5 \times 10^6 \pm 5.9 \times 10^6$ neutrophils after 4 hours, four times more than in animals injected intraperitoneally with saline ($12.0 \times 10^6 \pm 1.8 \times 10^6$ accumulated neutrophils). Intravenous injection of 200 μg of anti–PECAM-1 at the time of glycogen instillation blocked the increase in neutrophil recruitment, whereas intravenous injection of nonimmune rabbit IgG had no blocking ability (Fig. 2A). Repeated intravenous injections of a Fab preparation of anti–PECAM-1 blocked by 75% ($n = 5$, $P < 0.05$) glycogen-induced peritoneal neutrophil accumulation. Circulating white blood cell counts were monitored at 30-min intervals after antibody injection and showed no significant changes from base line counts.

The second in vivo model of neutrophil accumulation in rats involved deposition of IgG immune complexes in the lung. White blood cell emigration is quantitated by measurement of the number of neutrophils retrieved from the bronchoalveolar lavage (BAL) fluids after 4 hours. This is a quantitative reflection of neutrophil recruitment into the lungs (*13*). In this model, 67 μg of $F(ab')_2$ anti–PECAM-1 was infused intravenously at 2.5, 3.0, and 3.5 hours after immune complex injury was initiated (*10, 13*). Under these conditions, blood neutrophil counts at time $t = 0$ and $t = 4$ ($n = 4$) were $3.15 \times 10^6 \pm 0.19 \times 10^6$ per microliter and $3.41 \times 10^6 \pm 0.59 \times 10^6$ per microliter, respectively. In negative controls, the BAL fluid yielded $0.16 \times 10^6 \pm 0.03 \times 10^6$ neutrophils. In animals with immune complex deposition, the yield was $9.27 \times 10^6 \pm 1.87 \times 10^6$ neutrophils (Fig. 2B). Under conditions of anti–PECAM-1 treatment, $2.30 \times 10^6 \pm 0.38 \times 10^6$ neutrophils were retrieved, which indicates a 75% reduction ($P = 0.006$) in neutrophil accumulation. Thus, animals treated with anti–PECAM-1 have reduced recruitment of neutrophils. This effect was not due to nonspecific binding of an $F(ab')_2$ antibody to rat endothelium. With the use of the same protocol, the CL37 E-selectin $F(ab')_2$ antibody that reacts with rat endothelial cells did not reduce the accumulation of neutro-

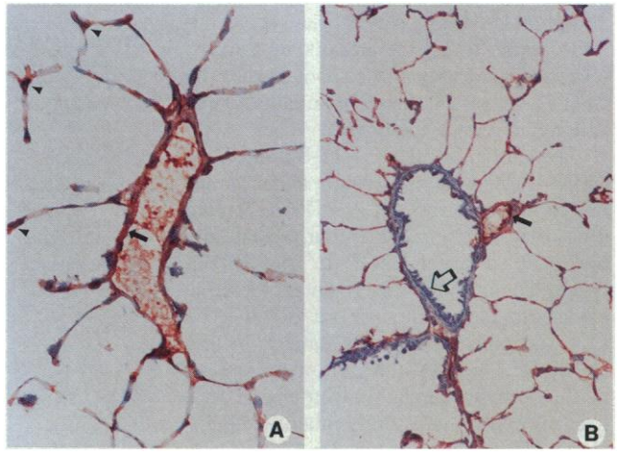

Fig. 1. Immunoperoxidase analysis of frozen sections of rat lung. The anti–PECAM-1 was used for immunohistochemical analysis on frozen sections of normal rat lung (*10*). Positive-staining tissues appear red. (**A**) High-power view (×250), demonstrating heavy venular endothelial staining (solid arrow) and capillary staining for PECAM-1 (arrowheads). (**B**) Lower power view (×100), again showing venular endothelial staining (solid arrow) and the absence of bronchial epithelial staining (open arrow).

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phils in the rat peritoneal or IgG immune complex model of lung inflammation (*10*).

To determine the extent to which anti–PECAM-1 might affect chemotaxis, we collected neutrophils from rat blood in the usual manner (*14*) and analyzed them in vitro for chemotactic responses to zymosan-activated rat serum (ZRAS) (*15*). Thirty times more neutrophils migrated through a Boyden-type chamber when 10% ZRAS was the chemoattractant. The chemotactic responses to ZRAS in the presence of either nonimmune rabbit IgG or rabbit anti–PECAM-1 at doses from 1 to 10 μg/ml were not significantly different. Anti–PECAM-1 or control IgG in the absence of ZRAS did not induce chemotaxis. These data suggest that although anti–PECAM-1 reacts weakly with a surface epitope on rat neutrophils, this interaction does not affect chemotactic responses nor blood neutrophil counts.

In a third approach to study the role of PECAM-1 in neutrophil transmigration, we transplanted normal human skin onto mice with severe combined immunodeficiency disease (SCID) (*16*). In this model, the vasculature of the transplanted skin retains its human phenotype and supports an inflammatory reaction. Each SCID mouse (n = 18) was grafted with two pieces of human foreskin obtained from the same donor (*16*). After 4 weeks, one graft in each mouse was injected with saline and the other with human recombinant tumor necrosis factor–α (TNF-α) (2000 U). Immediately after the intradermal injections, the animals received intravenous infusions of either saline (n = 6), nonimmune rabbit IgG Fab fragments (n = 3), or Fab fragments prepared from rabbit anti–PECAM-1 (n = 9). Circulating leukocyte counts were monitored and found to be unchanged as a result of these infusions.

The grafts were then harvested 4 hours after the intradermal injections and immunostained with antibody to mouse CD11b in order to identify leukocytes. The number of leukocytes present per square millimeter in the dermal interstitium was assessed. In animals injected intravenously with saline or control Fab fragments, TNF-α induced a four- to fivefold increase in the number of white blood cells migrating into the dermis compared to those mice injected with intradermal saline (P < 0.05) (Fig. 3A). In contrast, animals injected intravenously with anti–PECAM-1 Fab fragments showed only a 30% increase in the numbers of leukocytes accumulating in graft sites after local injection of TNF-α. This number was not significantly different from the number of leukocytes appearing in the saline-injected transplanted skin sites and was significantly less than the number of cells accumulating in the TNF-α–injected grafts of control animals (P < 0.01). In animals that received intravenous saline or irrelevant Fab, the interstitial areas of skin showed the diffuse presence of $CD11b^+$ cells (consistent with a neutrophil phenotype) after intradermal TNF-α injection (Fig. 3B). This is in contrast to mice that received anti–PECAM-1 Fab fragments intravenously and TNF-α intradermally, where leukocytes appeared to be primarily trapped within the vessels of the dermal microvasculature (Fig. 3, C and D). Similar leukocyte-laden vessels were infrequent in animals treated with control Fab fragments after local TNF-α injection (Fig. 3B).

Endothelial cell–neutrophil interactions are thought to occur in the context of an "adhesion cascade" in which cell adhesion molecules and chemoattractants are expressed in a programmed and sequential manner (*17, 18*). Selectins have been implicated in the earliest, relatively transient adhesions ("rolling"). Subsequent firm adhesion is achieved by activation of leukocyte integrins, which results in their interactions with endothelial

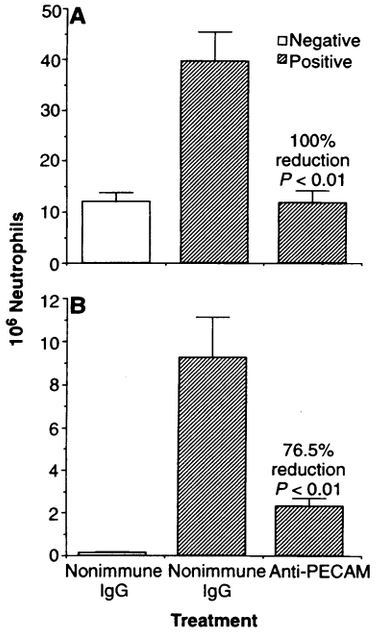

Fig. 2. Effect of anti–PECAM-1 on neutrophil emigration in vivo. (**A**) Effect of intact anti–PECAM-1 (200 μg, injected intravenously) on oyster glycogen–induced (25 ml of a 0.1% solution) neutrophil accumulation in the peritoneal cavity of male Long Evans rats (300 to 350 g). (**B**) The effect of anti–PECAM-1 on neutrophil emigration into the alveolar compartment of rat lungs after deposition of IgG immune complexes. These models have been described (*10, 13*). Open bars, untreated (negative control) animals; hatched bars, animals treated with intraperitoneal glycogen (A) or intratracheal IgG (B); error bars, SEM.

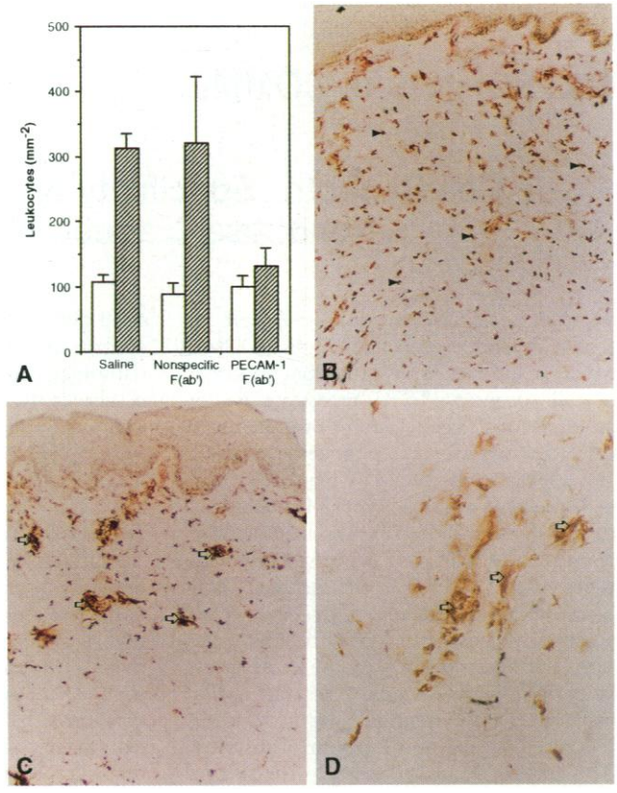

Fig. 3. Effect of anti–PECAM-1 on the transmigration of neutrophils across human endothelium. Human foreskin was grafted onto SCID mice (*16*). After 4 weeks, the grafts were injected intradermally with either saline (open bars) or 2000 U of human TNF-α (hatched bars). (**A**) Animals received intravenous injections of saline, 200 μg of control Fab fragments, or 200 μg of anti–PECAM-1 Fab fragments, and the number of intradermal leukocytes was measured. Immunohistochemical staining of TNF-α–injected skin grafts was done with a murine-specific CD11b antibody (*16*) to identify leukocytes. P < 0.05 in the first two sets of bars; P is not significant in the third. (**B**) A low-power view (×100) of a graft from an animal injected intradermally with TNF-α and intravenously with control Fab. There is diffuse infiltration of leukocytes (arrowheads) with little accumulation within the vascular wall. (**C** and **D**) Grafts (×100 and ×250 magnification, respectively) are from an animal injected intradermally with TNF-α and intravenously with anti–PECAM-1 Fab. Leukocytes appear trapped within the dermal vessel wall (open arrows). Relatively few white blood cells have transmigrated into the dermal interstitium.

adhesion molecules, namely ICAM-1 and ICAM-2. The mechanisms underlying the next step, transendothelial migration, are less clear but are assumed at the very least to involve a response to chemotactic stimuli. Our studies, along with other in vitro work (9), demonstrate that inhibition of PECAM-1 function can block the emigration of neutrophils and thus define the requirement for an additional cell adhesion molecule, PECAM-1, in the recruitment of neutrophils into inflammatory sites. It appears that this effect requires at least endothelial cell PECAM-1 because the anti–PECAM-1 used to block leukocyte transmigration in the human-SCID chimera model does not react against PECAM-1 on murine leukocytes (16). Although the mechanism by which PECAM-1 facilitates white blood cells through the endothelium is unresolved, blocking PECAM-1–mediated transmembrane migration of white blood cells may offer another target for therapeutic intervention in the treatment of inflammatory disorders.

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TECHNICAL COMMENTS

T Cell Receptor Specificity and Diabetes in Nonobese Diabetic Mice

In their search for the role of T cells in insulin-dependent diabetes mellitus (IDDM), Myra A. Lipes *et al.* (*1*) state that an intact T cell receptor (TCR) repertoire is not required for the generation of pancreatic β cell destruction in nonobese diabetic (NOD) mice. If IDDM is mediated by T cells, a single autoreactive TCR could lead to this disease. Therefore the key question is either, "What TCR genes are preferentially used?" or "Does T cell specificity play a role at all?" The report by Lipes *et al.* apparently does not address the first question, but does suggest that the TCR specificity of individual lymphocytes may not be essential for the amplification of the cell's lesions or for the development of insulitis. This finding seems to relate to the second question, yet their results do not convincingly support it.

Lipes *et al.* found that transgenic NOD mice bearing nondisease-related T cell receptor α and β subunit transgenes developed diabetes similar to that developed by control nontransgenic mice in terms of pathology and kinetics (*1*). T cell receptor α subunit transgene was transcribed, and β subunit transgene was expressed on the cell surface, which resulted in allelic exclusion on the endogenous TCRβ subunit gene locus (*1*). However, the expression of the TCRα subunit transgene (as determined by the amount of protein) is not shown; the presence of transcripts does not guarantee its expression on the cell surface. Therefore, it is possible that allelic exclusion on the TCRα endogenous gene locus did not occur (*1*). As a result, autoreactive TCRs may have been produced from the gene rearrangement. Another study suggests (*2*) that even though α transgene is expressed on the cell surface of T lymphocytes, rearrangement on the endogenous TCRα locus could still occur if T lymphocytes (bearing the TCR encoded by the transgenes) cannot be positively selected during their development. Lipes *et al.* do not address the question of whether the TCR-encoded transgene was compatible with the major histocompatibility complex (MHC) molecules of NOD mice, and thus, whether T cells bearing this TCR could be positively selected. Moreover, information about the specificity of T cells carrying transgenic TCR molecules was not provided. Despite the fact that both α and β subunit transgenes were derived from nondisease-related TCRs, the specificity after pairing them together is not known. Thus, one cannot rule out the possibility that T cells bearing transgene-encoded TCR could attack β cells of pancreas islets; this could be another source for the TCR specificity required for disease generation.

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Research on juvenile diabetes and the NOD mouse model on which it is based are challenged in the report by Lipes *et al.* (*1*). They found that NOD mice expressing one random pair of transgenes for the α and β chains of the TCR (consisting of the α chain from an anti–L^d $CD8^+$ clone and the β chain from an anti–chicken ovalbumin $CD4^+$ clone) have lymphocytic infiltration of their pancreas and the same incidence of diabetes as the nontransgenic NOD mice. Lipes *et al.* suggest that the T cells of these transgenic mice bear exclusively the transgenic TCRαβ and conclude that "the TCR specificity of individual lymphocytes may not be essential." In other words, T cells, irrespective of the specificity of their TCR, are driven to participate in the selective destruction of the β cells that produce insulin inside the islets of Langerhans. The supposition that T cells could be involved in immune responses where their TCR is not engaged has major theoretical implications that are not discussed in the paper. How, for example, could tolerance to self antigens be imparted if the antigen specificity of TCRs could be bypassed for T cell activation?

There is a simple way to account for the results described by Lipes *et al.* without conflicting with basic immunological concepts or with the widely accepted view that diabetes results from a T cell–mediated autoimmune process specifically targeting β cell antigens. When a pair of transgenic rearranged α and β