Vascular Adhesion Protein-1 Is Involved in Both Acute and Chronic Inflammation in the Mouse

Marika Merinen, Heikki Irjala, Marko Salmi, Ilkka Jaakkola, Arno Hänninen, and Sirpa Jalkanen

From the MediCity Research Laboratory and the Department of Medical Microbiology, University of Turku and National Public Health Institute, Turku, Finland

Vascular adhesion protein-1 (VAP-1) is an endothelial molecule that possesses both adhesive and enzymatic properties *in vitro*. So far, however, elucidation of its *in vivo* function has suffered from the lack of function-blocking reagents that are suitable for use in animal models. In this work we produced monoclonal antibodies against murine VAP-1 and characterized them using *in vitro* binding assays. We then examined whether the antibodies could prevent leukocyte migration in *in vivo* inflammation models, including two acute models (peritonitis induced with proteose peptone and interleukin-1 and air pouch inflammation enhanced by CCL21) and one chronic model (autoimmune diabetes in nonobese diabetic mice). Antibodies 7-88 and 7-106 inhibited migration of granulocytes and monocytes in both acute models of inflammation. Strikingly, antibody 7-88 significantly prevented diabetes in a subset of nonobese diabetic mice. The results show for the first time that in mouse models of inflammation, VAP-1 mediates leukocyte trafficking to sites of inflammation and thus is a potential target for anti-inflammatory therapies. (*Am J Pathol* 2005, 166:793–800)

During physiological lymphocyte recirculation lymphocytes leave the blood by binding to high endothelial venules (HEVs)¹ in an adhesion cascade involving several lymphocyte molecules and their endothelial counterparts. Moreover, leukocytes use similar mechanisms when entering the sites of inflammation.² Vascular adhesion protein (VAP)-1 is one of the endothelial molecules participating in the adhesive events between leukocytes and the vascular wall.³

Monoclonal antibodies against human VAP-1 have existed more than 10 years and they have been invaluable

in discovering the function of VAP-1. VAP-1 is a heavily sialylated homodimeric glycoprotein of 180 kd present in endothelial cells, smooth muscle cells, adipocytes, and in follicular dendritic cells.³ Structurally it belongs to enzymes called semicarbazide-sensitive amine oxidases that deaminate primary amines in a reaction producing hydrogen peroxide, aldehyde, and ammonia. *In vitro* studies have indicated that the enzyme activity is associated with the adhesive properties of VAP-1 and that a lymphocyte surface molecule most likely acts as a substrate for VAP-1. It has been proposed that this enzymatic reaction results in the formation of a transient Schiff base, via which a lymphocyte transiently adheres to endothelium during the multistep adhesion cascade.¹

In vitro studies using the above-mentioned monoclonal antibodies against human VAP-1 have indicated that VAP-1 mediates lymphocyte binding to HEVs and granulocyte adhesion to vasculature at sites of inflammation such as reperfusion injury connected to myocardial infarction.³ Certain anti-human VAP-1 antibodies cross-react with dog, pig, and rabbit VAP-1 and studies performed with them in these species have shown that on inflammation VAP-1 is rapidly translocated to the endothelial cell surface from intracellular sources.⁴ However, therapeutic studies have only been performed using rabbit peritonitis (4 hours) as an experimental model.⁵ Despite several previous attempts we have not been able to produce function-blocking antibodies against mouse VAP-1 and therefore, evaluation of the *in vivo* significance of VAP-1 in well-characterized mouse models of inflammation has not been performed. Here we report production of suitable anti-mouse VAP-1 reagents and, for the first time, demonstrate the *in vivo* involvement of VAP-1 in monocyte- and lymphocyte-dominated inflammations.

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Address reprint requests to Dr. Sirpa Jalkanen, MediCity Research Laboratory, Tykistökatu 6A, 20520 Turku, Finland. E-mail: sirpa.jalkanen@utu.fi.

Materials and Methods

Mice

Nonobese diabetic mice (NOD) (purchased from Bomholtgård, Ry, Denmark) and Balb/C (local colony) mice were bred and maintained under specific pathogen-free conditions in the Central Animal Laboratory of the Turku University. Cumulative incidence of diabetes in our colony reaches 70% in female mice. NOD mice were used at the ages specified for each experiment. Balb/C mice were used between 6 to 8 weeks of age. The local ethical committee approved the experimental procedures.

Antibodies

To produce monoclonal antibodies against murine VAP-1 rats were immunized to footpads with a suspension containing minced preparations of vessels that exit from mouse lymph nodes and incomplete Freund's adjuvant three times with 1-week intervals. The vessels were excised from the nodes under a stereomicroscope. Thereafter, the popliteal lymph nodes were collected and the lymphocytes fused with SP2/0 myeloma cells. Hybridomas were screened using frozen sections of mouse small intestine and peripheral lymph nodes and hybridomas producing antibodies that had an endothelial staining pattern were selected for further analyses and subcloning. The 7-88, 7-106, and 7-188 antibodies (all rat IgG2b) demonstrated reactivity against mouse VAP-1 when tested with VAP-1-transfected Chinese hamster ovary (CHO) cells (see below) but recognized different epitopes of the VAP-1 molecule.

R-phycoerythrin-conjugated antibodies against CD8 and CD4 were from Caltag Laboratories (Burlingame, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD11a (LFA-1), CD44, L-selectin (CD62L, MEL-14), $\alpha 4$ (CD49d), CD45RB, and rat IgG_{2a} were from PharMingen (San Jose, CA). Monoclonal antibodies (mAbs) Hermes-1 (clone 9B5 against human CD44), 3G6 (against chicken T cells), JG2.10 (against human VAP-1, kind gift from E. Butcher, Stanford University, Stanford, CA) and HB-151 (against human HLA-DR5; American Type Culture Collection, Rockville, MD) were used as isotype-matched control antibodies. Antibodies used in *in vivo* studies were concentrated from hybridoma supernatants grown in serum-free media with ammonium sulfate precipitation. The second stage antibodies were FITC-conjugated anti-rat IgG (Sigma-Aldrich Chemie GmbH, Germany) and peroxidase-conjugated rabbit anti-rat immunoglobulins (DAKO A/S, Glostrup, Denmark).

Flow Cytometric Analyses

Mouse VAP-1-transfected CHO (expressing mouse VAP-1)⁶ and mock-transfected CHO cells (0.5×10^6 cells/sample) were stained with anti-mouse VAP-1 antibodies (7-88, 7-106, 7-188) or control antibody (3G6) (100 μ l of supernatant per sample) and FITC-labeled anti-rat IgG secondary antibody containing 5% normal mouse serum.

For the epitope mapping mouse VAP-1-transfected CHO cells were first incubated with 7-88, 7-106, 7-188 (each 100 μ g/ml) antibodies or a polyclonal antibody against $\beta 1$ -integrin (recognizing $\beta 1$ -integrin on CHO cells) and after three washes FITC-conjugated 7-88, 7-106, and 7-188 (50 μ g/ml) were added in different combinations. The antibodies were conjugated to FITC (Sigma) and purified after overnight dialysis with PD-10 columns (Amersham Pharmacia Biotech, Sweden).

Lymphocytes isolated with a glass homogenizer from mesenteric lymph nodes and the spleen and filtrated to obtain single cell suspensions were stained either with phycoerythrin-conjugated anti-CD8 or CD4 and FITC-conjugated antibodies against CD11a, CD44, L-selectin, $\alpha 4$, CD45RB, or rat IgG_{2a} for flow cytometry (FACScan; BD Biosciences, San Jose, CA). Because of their low number pancreatic lymph node lymphocytes were only stained with phycoerythrin-conjugated anti-CD4 and -CD8 and FITC-conjugated anti-L-selectin, anti- $\alpha 4$, and rat IgG_{2a}. The results were analyzed using CellQuest software (BD Biosciences).

Immunohistochemistry

Frozen tissue sections from pancreas, lung, liver, kidney, spleen, skeletal muscle, gut, heart, and thymus were incubated with anti-mouse VAP-1 antibodies (7-88, 7-106, 7-188) or control antibody (Hermes-1) for 20 minutes at room temperature. All first stage antibodies were used as hybridoma supernatants. After two washings with phosphate-buffered saline (PBS) the sections were incubated with peroxidase-conjugated rabbit anti-rat Ig secondary antibody in PBS containing 5% normal mouse serum. Sections were washed again and 3'3'diaminobenzidine hydrochloride in PBS containing 0.03% hydrogen peroxide was added for 3 minutes. Finally the sections were counterstained with hematoxylin.

Frozen pancreatic tissues cut from female NOD mice (at the age of 2, 5, 8, and 11 weeks) and normal BALB/c mice (at the age of 2 and 11 weeks) treated with intravenous injection of 7-88 or negative class-matched control antibody (HB-151, 100 μ g/mice) were stained after acetone fixation with FITC-conjugated anti-rat IgG secondary antibody containing 5% normal mouse serum. At each age group two to three mice per strain were studied. Stained sections were mounted with Prolong Antifade (Molecular Probes, Eugene, OR) and coverslips.

In Vitro Adhesion Assay

Stamper-Woodruff-type of frozen section assays were performed as described earlier.⁷ Briefly, the lymph nodes used for the adhesion assays were the draining lymph nodes collected from mice previously injected into their footpads with Freund's incomplete adjuvant and normal peripheral lymph nodes. Pancreata were collected from diabetic NOD mice. Lymphocytes were isolated with a glass homogenizer from mesenteric lymph nodes and filtered to obtain a single cell suspension. Thereafter, they were incubated on freshly cut (8 μ m thick) frozen sec-

tions of lymph nodes and pancreata, which had been pretreated for 30 minutes with anti-mouse VAP-1 antibodies, 7-88, 7-106, 7-188, or control antibodies 2E8 (a non-function blocking antibody against an unknown molecule on mouse endothelial cells) or HB-151 under rotatory conditions (60 rpm) for 30 minutes at +7°C. All antibodies were used at a concentration of 50 µg/ml. After incubation adherent cells were fixed with 1% glutaraldehyde and cells bound to HEVs (or other venules in pancreata) were counted single blind under dark-field microscopy. The results are presented as percentage of maximal binding (100% = the number of bound cells in the presence of the control antibody) ± SEM.

In Vivo Models

Peritonitis

Inflammation was induced by a 1-ml intraperitoneal injection of PBS containing 5% proteose peptone and 10 ng of interleukin-1 α . One hour later the mice were treated with an intravenous injection of antibodies [200 µg of mAbs 7-88 (four mice), 7-106 (five mice), 7-188 (five mice), HB-151 (five mice), or pooled anti-VAP-1 antibodies (100 µg of each mAb per mouse, eight mice)]. In addition, to analyze the dose effect, three mice were treated with 50 µg of 7-106. Antibody treatment was repeated 4 hours later. At 18 hours after the induction of inflammation cells were collected from the peritoneal cavity by washing it with 10 ml of RPMI containing 5 U/ml heparin (Løvens Kemiske Fabrik, Denmark) and counted. Leukocyte subtypes were analyzed from lavage fluid smears after Diff-Quick (Reastain; Reagen, Finland) stainings.

Air Pouch Inflammation

Air pouches were established on the backs of mice (6 to 8 weeks old) with a subcutaneous injection of 5 ml of filtered air (Millex-GV filter unit; Millipore, Ireland). Injection was repeated on the next day with 3 ml of filtered air. On the third day 1 ml of RPMI containing CCL-21 (1 µg/ml, SLC, mouse 6Ckine; R&D Systems, Minneapolis, MN) and bovine serum albumin (50 µg/ml) were injected into the pouches. At 5 and 9 hours after this injection the mice (five mice in each group) were treated with antibodies as described for peritonitis. Cells were collected from pouches 23 hours after the CCL-21 injection with 5 ml of RPMI containing 5 U/ml of heparin and counted. Also leukocyte subtypes were analyzed from Diff-Quick-stained lavage fluid smears.

Diabetes

Female NOD mice were treated with intraperitoneal injections of monoclonal antibodies twice per week from 3 weeks of age until 30 weeks of age or until diabetes occurred. Before injections the mAbs were sterile-filtered through the Millex-GV filter unit (Millipore). Each injection consisted of 100 µl of anti-mouse VAP-1 antibody (100

µg of 7-88, 21 mice), control antibody (100 µg of HB-151, 15 mice), or 100 µl of PBS (14 mice).

Urinary glucose (Glukotest; Roche Diagnostics GmbH, Germany) was measured weekly and if the glucose level had elevated the blood glucose levels were measured (MediSense Precision Xtra Plus; Abbott Laboratories MediSense, UK). Blood glucose values greater than 14.3 mmol/L were considered diabetic.

In another set of experiments, female NOD mice were treated with anti-VAP-1 (7-88, eight mice) and control (HB-151, eight mice) antibodies twice per week from 3 weeks of age until 13 weeks of age as described above for the long-term treatment. After this 10-week treatment cell suspensions were made from mesenteric and pancreatic lymph nodes and spleens for further phenotypic analyses as described above.

Statistical Analyses

Analysis of variance with Bonferroni's multiple comparison test in peritonitis and air pouch models, Kaplan-Meier and log rank in diabetes, and paired *t*-tests in *in vitro* adhesion assays were used for statistical analyses.

Results

Antibodies 7-88, 7-106, and 7-188 Recognize the Mouse VAP-1 Molecule

Immunizations of rats with mouse vessels resulted in generation of three hybridomas, which produced mAbs with VAP-1-like staining patterns in preliminary immunohistological analyses with mouse small intestine. To confirm that these mAbs recognize mouse VAP-1 molecule we first stained stably transfected CHO cells expressing mouse VAP-1 and mock-transfected CHO cells with these antibodies. CHO cells expressing mouse VAP-1 were positively stained with all these new antibodies (7-88, 7-106, and 7-188) but remained negative when stained with the control antibody. In addition, mock-transfected CHO cells were negative with these mAbs (Figure 1). In epitope mapping, these antibodies recognized different but overlapping epitopes of VAP-1, because incubations with 7-88, 7-106, and 7-188 but not with anti- β 1 integrin partially inhibited staining of the VAP-1-transfected CHO cells with other FITC-conjugated anti-VAP-1 antibodies (data not shown).

All these antibodies produced a similar staining pattern when various mouse organs (pancreas, lung, liver, kidney, spleen, skeletal muscle, small intestine, heart, thymus, and lymph nodes) were tested. However, the intensity of the stainings slightly varied, most likely reflecting different affinities of the antibodies and/or epitope availability. The antibodies stained HEVs in lymph nodes and a subset of vessels in all organs studied. Moreover, smooth muscle cells and adipocytes were positive. In a striking contrast to human VAP-1⁸ sinusoidal endothelial cells of the mouse liver were practically negative for VAP-1. Examples of the expression pattern of mouse VAP-1 in different organs are shown in Figure 2 using 7-88.

Figure 1. Reactivity of anti-VAP-1 antibodies against recombinant VAP-1. Antibodies (7-88, 7-106, 7-188, and a negative control antibody) were used to stain both CHO cells transfected with a cDNA encoding mouse VAP-1 or with vector only (CHO mock). The x axis is the fluorescence intensity in a logarithmic scale and the y axis is relative number of cells. The experiment has been repeated four times.

7-88 Anti-mVAP-1 mAb Inhibits Lymphocyte Adhesion *in Vitro*

To test whether any of the new monoclonal antibodies have function-blocking properties *in vitro* adhesion assays were performed. In these assays lymph node sections were treated with 7-88 and showed ~50% reduction in lymphocyte binding ($P = 0.009$). A statistically significant effect was also found with 7-106, although it only inhibited binding by 12%. 7-188 did not show any inhibition of the binding (Figure 3). We also tested the ability of 7-106 to inhibit lymphocyte binding to vasculature in the inflamed pancreas. In these assays 7-106 showed 55% inhibition of binding both to vessels within and outside the islets (Figure 3).

VAP-1 Is Involved in Granulocyte and Monocyte Migration to Sites of Inflammation

We selected acute peritonitis as a model of a granulocyte-dependent acute inflammation. In our model, mice

Figure 2. VAP-1 is present in a subpopulation of vessels in several organs and in smooth muscle cells. Frozen sections of the indicated organs were stained with 7-88 anti-VAP-1 antibody or a negative control antibody as the first stage reagent followed by peroxidase-conjugated anti-rat Ig. **Arrows** point out some positive vessels. Sm, smooth muscle. Original magnifications, $\times 200$.

treated with the control antibody had $6.0 \pm 0.6 \times 10^6$ (mean $\pm$ SEM) cells in the peritoneal lavage and the most frequent individual cell type among them was granulocytes. Intravenous treatment with each anti-VAP-1 antibody individually or in combination decreased the number of leukocytes in the peritoneal cavity. Treatment with 7-106 reached best inhibition ($P < 0.01$). The inhibition was dose-dependent because 200 μg per mouse gave a 36% inhibition and 50 μg gave a 24% inhibition (Figure 4a and data not shown). Treatments with 7-88 and the pool of the three mAbs also showed inhibition ($P < 0.05$, Figure 4a). When granulocytes were counted separately, their number in the lavage fluid was decreased 51% by 7-106 ($P < 0.01$, Figure 4b).

To especially analyze the effect of anti-VAP-1 antibodies on monocyte migration to sites of inflammation an air pouch inflammation model was used because the majority of the infiltrating leukocytes in this model belongs to monocyte lineage. In this model, control antibody-treated animals had $1.8 \pm 0.3 \times 10^6$ (mean $\pm$ SEM) cells in the pouches and 49% of them belonged to the monocyte/macrophage lineage, 43% were granulocytes, and the rest were lymphocytes. Antibody treatment with 7-106 decreased the total number of all infiltrating cells by 60% ($P < 0.01$), whereas treatment with 7-88 or the pool of all monoclonals decreased the number by 50% ($P < 0.05$, Figure 5a). The slightly better efficiency of 7-106 compared to the pool of antibodies is most likely because of the dosing because 7-106 was given 200 μg per mouse when injected alone, whereas in the pool its amount was 100 μg per mouse. When monocytes were analyzed

Figure 3. VAP-1-dependent inhibition of lymphocytes to lymph node HEVs and to vasculature in inflamed pancreas. Lymphocyte binding to lymph node HEV (a) and vessels in inflamed pancreata (b) was measured using a Stamper-Woodruff-type of an adhesion assay. The results are presented as percentage of control binding (set as 100% in the presence of a negative control antibody) $\pm$ SEM. c: An example of lymphocyte binding to two HEV-like vessels (outlined by dashed lines) in an inflamed pancreatic islet (the border of the islet is outlined with a dotted line). Arrows point out some of the bound lymphocytes (dark round cells). Original magnifications, $\times 200$.

separately 7-106 inhibited their migration by 66% ($P < 0.001$, Figure 5b). The antibody pool ($P < 0.001$) and 7-88 ($P < 0.05$) also caused significant inhibition. Migration of all leukocyte populations to inflamed air pouches was impaired (for example lymphocyte trafficking was diminished by 40% and granulocyte migration by 53% with the anti-mVAP-1 antibody pool).

VAP-1 Expression in Mouse Pancreas during the Development of Diabetes

We have earlier quantified VAP-1 in pancreatic vasculature during the development of diabetes in mice.⁶ However, the antibody used for those studies was made against a fusion protein and it only recognized the denatured VAP-1 protein and thus did not allow detection of the native protein nor testing the functionality of VAP-1. Therefore, these new antibodies gave us an opportunity to analyze whether VAP-1 is translocated to the endothelial cell surface during the development of diabetes.

Figure 4. VAP-1 contributes to granulocyte trafficking in inflamed peritoneum. Proteose peptone and interleukin-1 α were used to induce peritonitis and at 1 hour and 4 hours thereafter anti-VAP-1 or control mAbs were administered intravenously. The lavage fluid was collected at 18 hours after induction and the infiltrated leukocytes and granulocytes were counted. The results are presented as percentages of the number of total leukocytes (a) and granulocytes (b) recovered from the mice treated with the control antibody (set as 100%) $\pm$ SEM.

Figure 5. VAP-1 inhibits leukocyte trafficking to inflamed air pouch. The mice were treated after onset of the inflammation with the indicated antibodies. At the end of the experiment the cells in the lavage fluid were collected and counted. The results are presented as percentages of the number of leukocytes (a) and monocytes (b) recovered from the mice treated with the control antibody (set as 100%) $\pm$ SEM.

Figure 6. VAP-1 is present on the surface of pancreatic vessel wall at an early age in NOD mice. The mice were intravenously injected with anti-VAP-1 antibody (7-88) or a negative control antibody. The bound antibody was detected in frozen sections using a FITC-conjugated second stage antibody. Original magnifications, $\times 400$.

When anti-mouse VAP-1 antibody (7-88) was injected intravenously to NOD mice at different ages and allowed to circulate for 30 minutes, and the bound antibody was detected in frozen sections made from the pancreata of these mice using a FITC-conjugated second stage reagent, VAP-1 was detected on the endothelial cell surface in pancreas already at 2 weeks of age. A similar staining pattern was detected in the pancreata of mice at 5, 8, and 11 weeks of age. No staining was detected in the pancreata of mice given a control antibody (Figure 6). However, VAP-1 expression on pancreatic endothelium is not unique to NOD mice, because BALB/c mice also have VAP-1 on their pancreatic vessels although to a lesser extent (data not shown).

Longstanding Anti-mVAP-1 Antibody Treatment Affects the Development of Diabetes

Next we wanted to evaluate whether a preventive antibody treatment started at the age of 3 weeks, before any drastic inflammatory changes have taken place in NOD mice, can prevent diabetes development. 7-88 was selected for this long-term treatment because of its efficacy in the *in vitro* assays and because of its availability. The first group of mice was treated twice per week for 6 months with either 7-88 antibody ($n = 13$) or PBS ($n = 14$). In the PBS group 11 of 14 mice became diabetic by the end of the treatment, whereas in the anti-VAP-1-treated group the corresponding ratio was 7 of 13. At the end of the follow-up time (1 year) the difference in the incidence between the groups remained approximately the same. To exclude the possibility that this difference in the incidence was simply because of a nonspecific effect of the antibody a second group of mice was treated in a similar manner except that a class-matched negative control mAb was used instead of PBS. In this experiment 8 mice received 7-88 anti-VAP-1 antibody and 15 animals received the class-matched control antibody for 6

Figure 7. VAP-1 is a target for the prevention of autoimmune diabetes. NOD mice were treated intraperitoneally for 6 months twice per week with anti-VAP-1 antibody (7-88) or an irrelevant class-matched control antibody and the incidence of diabetes was followed for 1 year. See Materials and Methods for further details.

months and they were then followed up until the age of 1 year. At the end of the follow-up time 10 of 15 mice had become diabetic in the group treated with the control antibody, whereas 3 of 8 mice treated with anti-VAP-1 became diabetic (Figure 7). If both of the experiments are combined treatment with anti-VAP-1 antibody prevented in a statistically significant manner the development of diabetes when compared to PBS treatment ($P = 0.017$).

Anti-mVAP-1 Antibody Treatment Slightly Changes L-Selectin Expression

To test whether anti-VAP-1 antibody treatment causes changes in relative number of lymphocyte subpopulations NOD mice were treated in a similar manner as in the long-term protocol except that the treatment duration was 10 weeks. There were no marked differences in the T:B cell or CD4:CD8 ratios. However, in control-treated animals L-selectin disappeared from the CD4- and CD8-positive cells in pancreatic lymph nodes whereas this down-regulation was incomplete in the anti-mVAP-1-treated group (Figure 8). In contrast, there was no difference in the expression level of L-selectin in splenic and mesenteric lymph node lymphocytes between the groups. Expression levels of CD11a, $\alpha 4$ integrin, CD44, and CD45RB were similar in T cells from mesenteric lymph nodes and in spleens of both groups. There was no difference in $\alpha 4$ expression on pancreatic lymph node lymphocytes between the groups.

Discussion

The most important finding of this work was that migration of different leukocyte subsets to sites of inflammation could be diminished both in acute and chronic conditions by targeting VAP-1. Although there has been a considerable amount of *in vitro* evidence concerning the role of VAP-1 in leukocyte trafficking in humans, *in vivo* efficacy studies using VAP-1 as a target have seriously suffered from the lack of suitable reagents to be used in different

Figure 8. L-selectin phenotypes after antibody treatments. Starting at the age of 3 weeks, NOD mice were treated for 10 weeks with 7-88 or a negative control antibody twice per week. At the end of the experiment the lymphocytes were isolated from the indicated organs and double stained. Each histogram is a pooled cell suspension of either anti-VAP-1-treated animals (eight mice) or control-treated ones (eight mice). The x axis is the fluorescence intensity in a logarithmic scale and the y axis is the relative number of cells. The **black line** is anti-VAP-1-treated animals and the **gray line** illustrates control-treated mice.

experimental disease models. Therefore, these present *in vivo* findings are encouraging and strongly suggest that VAP-1 can be used as an anti-inflammatory target also in human medicine.

The molecular interactions mediating leukocyte binding to vascular endothelial cells are evolutionarily well conserved.⁹ This can be realized from the fact that leukocytes from different species can bind to vascular endothelial cells of other species in *in vitro* assays. This was also experienced by us while trying to make monoclonal function-blocking antibodies against mouse VAP-1 using rats, because only 1 rat of 10 immunized with isolated vessels or purified antigen was capable of responding in the manner that provided us with the antibodies presented in this study. This indicates that the immunogenic epitopes between mouse and rat are shared to the extent that the immune system of most rats does not recognize them.

The enzymatic function of VAP-1 markedly contributes to the rolling and transmigration phase of leukocytes and the current model suggests that adhesive and enzymatic activities of VAP-1 take place sequentially and are thus part of the same cascade.¹ Therefore, the binding of leukocytes to VAP-1 can be blocked either with antibodies that inhibit the adhesion or small molecular inhibitors that block the enzymatic reaction. None of our anti-mouse antibodies were able to inhibit the enzymatic activity of VAP-1 (data not shown). Similarly, none of 15 different anti-human VAP-1 monoclonal antibodies that we have produced so far block the enzymatic activity of VAP-1. These results suggest that the important structures for enzymatic activity are not immunogenic. This is well understandable in the light that semicarbazide-sensitive amine oxidase activity is well conserved during the evolution from bacteria to humans.^{10–12}

The binding efficiency of lymphocytes to HEVs in frozen sections of lymphoid organs in Stamper-Woodruff-

type of *in vitro* assays reflects relatively well the homing capacity of lymphocyte subpopulations *in vivo*. The blocking effects of different antibodies in this assay have also successfully predicted the results of *in vivo* studies.^{13,14} In this present work the inhibition percentages of *in vitro* studies using 7-88 hold true *in vivo*. Interestingly, however, although 7-106 was a poor inhibitor of lymphocyte binding to lymph node HEV it showed the best inhibition *in vivo*. The mechanism behind this phenomenon remains to be elucidated.

Many molecules involved in the adhesion cascade between leukocytes and vascular endothelial cells have been successfully targeted in various *in vivo* animal models.¹⁵ When the treatments have been transferred to clinical trials, the results have been rather disappointing. However, recent results obtained by treating multiple sclerosis patients and Crohn's patients with anti- $\alpha 4$ integrin antibody and psoriasis with anti-CD11a antibody are most promising and indicate that antibodies against homing molecules can be beneficial in clinical medicine, if the target is correctly chosen.^{16–18} One of the reasons for poor success in adhesion-related therapies has been that in many animal models the antibody is given simultaneously at the time of induction of the inflammation. This possibility is rare in human medicine, in which the inflammation has usually already started before the patient seeks for medical help. For this work we chose very different models to test the feasibility of VAP-1 as an anti-inflammatory target. In the peritonitis model we started the antibody treatment only an hour later than induced the disease. In contrast, in the air pouch model the antibody was given clearly after the induction of the inflammation. The results obtained suggest that VAP-1 targeting is beneficial still after the onset of the disease. In the case of diabetes, it is important to start the treatment before the insulin-producing β cells have been irreversibly destroyed. This type of practice is at least partially feasible in clinics, where high-risk patients for diabetes (eg, the relatives of diabetic persons and those carrying a susceptible HLA type) can be followed up and screened for the existence of autoantibodies with high predictive value.

As anti-adhesive therapy against homing molecules carries the inherent disadvantage of potentially modulating the normal immune system and increasing susceptibility to infections, we wanted to test the long-term effects of anti-VAP-1 treatment on leukocyte subsets. Based on the results VAP-1 does not seem to markedly change the differential counts of leukocytes nor lymphocyte phenotypes. Also the fact that the majority of VAP-1 is stored within intracellular granules and translocated to the endothelial cell surface on inflammation suggests that targeting of it may not seriously impair the normal immune system. This view is also supported by the fact that despite long-term antibody treatment (6 months) no infections were discovered among the mice. On the other hand, the reduction of 50 to 60% in the number of migrating leukocytes to sites of inflammation by targeting VAP-1 most likely reflects the fact that a multitude of redundant molecules are involved in leukocyte extravasation and by blocking one it is practically impossible to completely

prevent the accumulation of leukocytes at sites of inflammation. The inhibition percentages obtained with anti-VAP-1 antibodies are well in line with percentages of blocking by antibody therapies targeting other adhesion molecules.^{19,20} However, in many situations, as for example in transplantation rejection and reperfusion injuries, 50% inhibition in leukocyte extravasation may be more than is needed to rescue the organ.

In conclusion, we used various inflammation models in the mouse and antibodies against different epitopes of murine VAP-1 to show that the targeting of functionally important epitope(s) of VAP-1 can achieve beneficial effects by inhibiting the accumulation of leukocytes at sites of inflammation.

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