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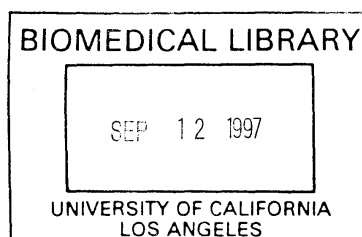
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Aims and Scope

Molecular Immunology is primarily devoted to the publication of immunological knowledge which can be delineated at the molecular level. Within this framework are included contributions concerned with the molecular analysis of the cellular components and processes which underlie the physiological behavior of cells involved in immune phenomena. **Papers which merely describe new monoclonal antibodies and/or epitope mapping will no longer be accepted unless they have a direct relevance to the understanding of the immune system.** The various forms of communications in *Molecular Immunology* will be as follows:

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HUMANIZED PORCINE VCAM-SPECIFIC MONOCLONAL ANTIBODIES WITH CHIMERIC IgG2/G4 CONSTANT REGIONS BLOCK HUMAN LEUKOCYTE BINDING TO PORCINE ENDOTHELIAL CELLS

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Abstract—Discordant xenografts surviving the initial hyperacute rejection phase may be subject to cellular rejection processes mediated by infiltrating leukocytes including T cells, NK cells and monocytes. The stable adhesion of these cell types to endothelial cells is due to the molecular interaction of the integrins VLA-4 and LFA-1 with their ligands vascular cell adhesion molecule (VCAM) and ICAM-1 present on the endothelial cells. Human VLA-4 binds to porcine VCAM, and blocking mAbs specific for porcine VCAM have been developed. We have localized the epitope of the anti-porcine VCAM blocking mAbs 2A2 and 3F4 to domains 1 and 2, respectively. Humanized antibodies (IgG4 isotype) were constructed from these anti-porcine VCAM antibodies and demonstrated to inhibit adhesion of Ramos, Jurkat and YT cells, as well as purified resting and activated human T cells, to porcine aortic endothelial cells (PAEC). These cell types express both LFA-1 as well as VLA-4, suggesting blockade of human VLA-4 interaction with porcine VCAM may alone be sufficient to significantly impair adhesion of human leukocytes to porcine endothelial cells. The chimeric anti-porcine VCAM (pVCAM) HuG4 antibodies promoted increased adhesion of Fc receptor (FcR) positive cells such as U937 monocytic cells to PAEC. In contrast, chimeric anti-porcine VCAM antibodies created using the CH1 and hinge region from human IgG2 and the CH2 and CH3 regions from human IgG4 (HuG2/G4 antibodies) inhibited binding of FcR positive cells to PAEC. These chimeric anti-pVCAM antibodies should allow delineation of the *in vivo* role of VLA-4/VCAM interaction in porcine-to-primate xenotransplants. Further, the design of the HuG2/G4 antibodies should render them efficacious in multiple settings requiring elimination of FcR binding. © 1997 Elsevier Science Ltd.

Key words: adhesion molecules, antibodies, Fc receptors, inflammation, xenotransplantation.

INTRODUCTION

Although much progress has been made in elucidating the molecular mechanisms of hyperacute rejection of xenotransplants (reviewed in Somerville and D'Apice, 1993), the mechanisms of cell mediated rejection of xenografts remain unclear. The cellular infiltrate observed 3 days following transplantation of guinea pig hearts into complement (C)-depleted rats is comprised predominantly of macrophages and natural killer cells, with low numbers of T lymphocytes present (Blakely *et al.*, 1994). Similarly, the cellular infiltrate present 6 days after transplantation of porcine fetal islet clusters into rats is comprised predominantly of macrophages with low numbers of T lymphocytes present primarily at the periphery of the

graft (Wallgren *et al.*, 1995). *In vitro* porcine aortic endothelial cells (PAEC) as well as antigen presenting peripheral blood cells can directly activate human CD4+ and CD8+ T lymphocytes (Rollins *et al.*, 1994a; Murray *et al.*, 1994; Yamada *et al.*, 1995). Thus the initial phases of cellular rejection of xenotransplants are likely to involve binding and activation of multiple types of leukocytes including not only T lymphocytes but also natural killer cells and monocytes.

The stable adhesion of lymphocytes, natural killer cells, and monocytes to endothelial cells is mediated predominantly by the interaction of the integrins LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) with their cognate ligands ICAM-1 and VCAM-1 on endothelial cells (Allavena *et al.*, 1991; Jonjic *et al.*, 1992). For both monocytes (Luscinskas *et al.*, 1994) and T lymphocytes (Alon *et al.*, 1995; Luscinskas *et al.*, 1995) the binding of VLA-4 to VCAM is an essential component of the *in vitro* adhesive interactions required for stable arrest under

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conditions of flow. *In vitro*, the transmigration of NK cells and monocytes across human umbilical vein endothelial cell (HUVEC) monolayers also utilizes both LFA-1/ICAM-1- and VLA-4/VCAM-dependent pathways (Bianchi *et al.*, 1993; Meershaert and Furie, 1994; Chuluyan *et al.*, 1995). *In vivo*, the interaction of LFA-1 with ICAM-1 may not be completely essential for extravasation of lymphocytes or monocytes since both of these cell types are present in inflammatory infiltrates of leukocyte adhesion deficiency patients who have a genetic deficiency in CD18 and thus lack integrins such as LFA-1 (Hawkins *et al.*, 1992). Finally, numerous studies have demonstrated the potential of blocking antibodies directed against LFA-1 (Issekutz, 1992; Scheynius *et al.*, 1993), VLA-4 (Issekutz, 1991; Yednock *et al.*, 1992; Chisholm *et al.*, 1993), ICAM-1 (Zeng *et al.*, 1994), or VCAM (Orosz *et al.*, 1993; Silber *et al.*, 1995) to inhibit the accumulation of lymphocytes at inflammatory sites in animals.

For xenogeneic cellular interactions it is not yet fully defined which adhesion molecule interactions will function across species barriers. It is likely that at least some xenogeneic receptor-ligand interactions will be non-functional. For example, mouse LFA-1 does not bind to human ICAM-1 (Johnston *et al.*, 1990). To explore the molecular basis of adhesive interactions between distinct classes of adhesion molecules on human and porcine cells we have cloned porcine E-selectin and VCAM-1 and have demonstrated that both are capable of mediating the adhesion of human leukocytes (Rollins *et al.*, 1994a; Mueller *et al.*, 1995). Further, we have developed murine monoclonal antibodies (mAbs) specific for porcine VCAM which block binding of human VLA-4 to porcine VCAM (Mueller *et al.*, 1995). The functional blocking activity of these antibodies suggests that they will be useful in suppressing the cellular response to a porcine xenograft. However, murine mAbs are not ideally suited for use in primates due to their short half-life and high immunogenicity (Khazaeli *et al.*, 1994). Here we have utilized antibody engineering strategies to create humanized anti-porcine VCAM antibodies that block adhesion of human natural killer cells, monocytes, and T lymphocytes to activated PAEC. Furthermore, we show the choice of human isotype has critical implications for the potential therapeutic use of humanized antibodies in xenotransplantation.

MATERIALS AND METHODS

Cells

Ramos, Jurkat and U-937 cells were obtained from the American Type Culture Collection, YT cells were the gift of Dr J. M. Wang (National Cancer Institute, Frederick, MD, U.S.A.), porcine aortic endothelial cells (PAEC) were obtained from Cell Systems (Kirkland, WA, U.S.A.) and 293-EBNA cells were obtained from Invitrogen (San Diego, CA, U.S.A.). U-937 cells were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS and 2 mM L-glutamine. Ramos and Jurkat cells were

maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 50 μ M β -mercaptoethanol, 100 units/ml penicillin and 100 μ g/ml streptomycin. YT were maintained in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. PAEC were maintained in medium A consisting of M199 supplemented with 20% heat-inactivated FBS, 0.05 mg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.), 20 mM HEPES and 0.1 mg/ml heparin, and were used within three passages. 293-EBNA cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 250 μ g/ml G418. Human T cells were purified as described previously (Mueller *et al.*, 1995).

Production of VCAM/Ig fusions

A plasmid containing the human IgG1 Fc region was obtained from Dr M. J. Lenardo (NIH, Bethesda, MD, U.S.A.). DNA fragments containing various domains of human or porcine VCAM were obtained by PCR amplification of appropriate templates and joined in frame to the human IgG1 Fc coding region using standard molecular biology techniques. The cassettes encoding the fusion proteins were ligated into the expression plasmid pAPEX-3 (Evans *et al.*, 1995a). VCAM/Ig fusion proteins were produced by transfection of 293-EBNA cells as described previously (Evans *et al.*, 1995a). Three days following transfection the media were harvested, centrifuged to remove debris, and stored at 4°C. For ELISA, PolySorp 96-well microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 100 ng/well goat anti-human IgG Fc (Sigma, St. Louis, MO, U.S.A.) in 0.1 M carbonate buffer, pH 9.6. The plates were then washed three times with PBS supplemented with 0.5% Tween 20 and incubated with blocking buffer (PBS supplemented with 0.05% Tween 20) at 37°C for 1 hr. After three further washes, various dilutions of conditioned media in blocking buffer were added and incubated at 37°C for 1 hr. Following three further washes, 2A2 or 3F4 mAb in blocking buffer was added and incubated for 1 hr at 37°C. After additional washing, peroxidase-conjugated goat anti-mouse IgG Fc (Sigma) diluted 1:2000 in blocking buffer was added and incubated at 37°C for 1 hr. After three final washes the plate was developed for 15 min with 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma). Reactions were stopped by addition of 1 M sulfuric acid. Quantitation was performed by measuring absorbance at 490 nm using a Bio-Rad model 3550 plate reader.

Production of chimeric antibodies

Variable regions were cloned from the 2A2 and 3F4 hybridomas by PCR amplification as described previously (Evans *et al.*, 1995a, 1995b). These sequences have been submitted to the EMBL/GenBank/DBBL under accession numbers U78799–U78802. The variable regions were joined to genomic DNA cassettes containing

either the intact human IgG4 constant regions or the first constant region and hinge linker region from human IgG2 followed by the second and third constant regions from human IgG4 using standard molecular biology techniques. Chimeric antibodies were produced in 293-EBNA cells and purified by chromatography on protein A Sepharose as described (Evans *et al.*, 1995a). Each chimeric antibody preparation was greater than 95% pure as judged by Coomassie staining of SDS polyacrylamide gels. F(ab')₂ was prepared by digestion of the antibodies with ficin (Pierce, Rockford, IL, U.S.A.) followed by protein A chromatography.

Flow cytometry

Cells used were obtained by trypsinization (PAEC and 293-EBNA) or centrifugation (Ramos, Jurkat, YT and U-937 cells). Harvested cells were washed once with 4°C PBS containing 1% heat-inactivated FBS (PBS/1) and aliquoted at 50 000–100 000 cells per well in 96-well V-bottom plates (Nunc). Cells were washed two additional times with PBS/1, and then incubated with PBS/1 containing 10 µg/ml primary Ab or PBS/1 alone for 30 min at 4°C. For some experiments the chimeric HuG4 or HuG2/G4 was centrifuged at $1 \times 10^5 g$ for 1 hr prior to addition to the cells. The cells were then washed twice with PBS/1 and resuspended in PBS/1 containing the appropriate FITC-conjugated secondary antibodies (1:200 dilution) for 30 min at 4°C. The cells were again washed twice with PBS/1 and resuspended in PBS. Cell fluorescence was determined using a Becton Dickinson FACSsort.

Adhesion assays

On day 0 PAEC were plated in medium A at a density of 10 000 cells per well in a 96-well flat bottom plate. On day 1 the PAEC were refed medium A supplemented with 1 µg/ml LPS (Sigma, *E. coli* 0111:B4). On day 2, human cells were resuspended in the appropriate growth medium supplemented with 10 µM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) and incubated at 37°C for 30 min. The cells were subsequently washed three times with growth medium and resuspended at 6×10^6 cells/ml in RPMI 1640 supplemented with 1% heat-inactivated FBS (RPMI/1). The PAEC were washed twice with RPMI/1 and refed 50 µl/well of RPMI/1 containing the indicated concentrations of antibodies. After adding calcein-labeled cells at a density of 3×10^5 cells/well (50 µl), the plate was centrifuged at 200g for 1 min and incubated at 37°C for 15 min. The nonadherent cells were removed by washing three to five times with RPMI/1. Adherent cells were lysed with HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Background fluorescence was determined from wells containing PAEC that did not receive calcein-labeled cells. Samples were performed in duplicate in each experiment. Adherence was determined by comparing the residual fluorescence concentration in each well to the input fluorescence con-

centration. In each experiment the specific fluorescence signal obtained in the absence of competing antibody was defined as 100%.

For adhesion assays using purified soluble pVCAM (Mueller *et al.*, 1995), PolySorp 96-well microtiter plates (Nunc) were coated overnight at 4°C with 200 ng/well purified soluble pVCAM in 0.1 M carbonate buffer, pH 9.6. After blocking the plate with RPMI 1640 supplemented with 10 mg/ml BSA (RPMI/BSA), the plates were incubated with RPMI/BSA containing antibody for 1 hr at 37°C. Subsequently, 3×10^5 calcein-labeled U-937 cells in RPMI/BSA were added to each well. The plate was centrifuged upright for 1 min at 200g and incubated at 37°C for 15 min. The plate was then centrifuged inverted at 200g for 3 min. Adherent cells were lysed by the addition of HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RESULTS

Identification of porcine VCAM domains recognized by blocking mAbs

Previously, we have identified two murine mAbs (3F4 and 2A2) directed against porcine VCAM (pVCAM) which block VLA-4 dependent adhesion of peripheral blood human T cells to PAEC. These two mAbs are specific for pVCAM and do not recognize human VCAM (hVCAM). Here an initial analysis of the epitope specificity of the 2A2 and 3F4 mAbs was performed using fusion proteins consisting of various domains of pVCAM or hVCAM joined to the Fc region of human IgG1. The VCAM/Ig fusion proteins were produced in 293-EBNA cells and assayed for binding of 2A2 and 3F4 mAbs by ELISA (Fig. 1). Both the 2A2 and 3F4 mAbs bound efficiently to proteins containing the first three domains of pVCAM. The 2A2 mAb bound to fusion proteins containing pVCAM domain 1 such as pD1+pD2 (porcine domain 1 plus porcine domain 2) and pD1+hD2 (porcine domain 1 plus human domain 2), but did not bind to fusion proteins lacking pVCAM domain 1 such as pD2+pD3, hD1+pD2, or hD1+hD2. Weak binding of the 2A2 mAb could also be detected to a fusion protein containing only porcine domain 1 (pD1) but not to fusion proteins containing only pD2 or pD3. Conversely, the 3F4 mAb bound to fusion proteins containing pVCAM domain 2, such as pD1+pD2, pD2+pD3, and hD1+pD2, but did not bind to fusion proteins lacking pVCAM domain 2, such as pD1+hD2 and hD1+hD2. Binding of the 3F4 mAb could not be detected to fusion proteins containing only a single pVCAM domain. The weak binding of 2A2 and 3F4 to fusion proteins containing only a single VCAM domain is similar to previously characterized blocking antibodies specific for human VCAM and ICAM-1 which require the presence of at least two adhesion molecule domains for binding (Pepinsky *et al.*, 1992; Renz *et al.*, 1994). Together, these results suggested the 2A2 mAb specificity is defined by

Leader	VCAM			HuG1	2A2	3F4	Anti-Fc
	D1	D2	D3		100	100	100
	D1	D2	D3		119	104	105
	D1	D2			92	87	95
		D2	D3		0	96	106
	D1	hD2			74	0	122
	hD1	D2			0	100	100
	hD1	hD2			0	0	102
	D1				5	0	96
		D2			0	1	100
			D3		1	0	115

Fig. 1. Binding of 3F4 and 2A2 mAbs to VCAM/Ig fusion proteins. Proteins consisting of domains 1 (D1), 2 (D2), or 3 (D3) from porcine VCAM (open boxes) or human VCAM (gray boxes) joined to the Fc region of human IgG1 (black boxes) were produced in 293-EBNA cells. In some constructs the pVCAM secretory leader sequence was replaced with the secretory leader sequence from human CD59 (striped boxes). The fusion proteins were captured by incubating diluted conditioned media in 96-well plates coated with goat anti-human IgG (Fc specific). After incubation with 2A2 or 3F4 mAb, bound murine mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific). The total amount of each fusion protein captured per well was quantitated using peroxidase-conjugated goat anti-human IgG (Fc specific). The absorbance signal obtained with the fusion protein containing the intact pVCAM domains 1, 2 and 3 was defined as 100% for each antibody. Binding of the 2A2 and 3F4 mAbs to the remaining fusion proteins is reported as a percentage of this value. Results shown are from a representative experiment of several performed.

pVCAM domain 1 whereas the 3F4 mAb specificity is defined by pVCAM domain 2.

Inhibition of human leukocyte binding to PAEC by chimeric anti-porcine VCAM mAbs

The use of murine antibodies in porcine-to-primate xenotransplantation models or ultimately in porcine-to-human xenotransplants poses several problems including a short half-life of the murine antibody plus development of an immune response directed against the murine antibody. These problems have been significantly reduced by the use of chimeric antibodies consisting of the murine variable regions joined to human constant regions (Khazaeli *et al.*, 1994). To create anti-pVCAM chimeric antibodies we cloned the variable regions from both 2A2 and 3F4. The murine variable heavy regions were joined to the human IgG4 constant region (HuG4) since this isotype does not activate complement (C) (Tao *et al.*, 1993), the predominant mediator of hyperacute rejection of xenografts. The murine variable light regions were joined to the human kappa constant region. Chimeric ch2A2 HuG4 and ch3F4 HuG4 antibodies were produced by coexpression of the chimeric heavy and light chains in 293-EBNA cells and tested for binding to pVCAM by FACS analysis using 293 cells expressing pVCAM (Fig. 2(A)). Both chimeric mAbs produced identical staining profiles as the corresponding murine mAbs, while neither chimeric mAb bound to untrans-

fectected 293 cells. Additionally, both the chimeric antibodies and murine mAbs produced identical FACS profiles using LPS-activated porcine aortic endothelial cells (Fig. 2(B)), confirming the chimeric antibodies retained the ability to bind to pVCAM expressed on PAEC.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to block the interaction between human VLA-4 and porcine VCAM was assessed by measuring inhibition of Ramos cell binding to activated PAEC (Fig. 3). The murine 3F4 mAb inhibited this binding by 90% when present at concentrations greater than 5 µg/ml. A control humanized antibody directed against human C5 (h5G1.1 HuG4) did not inhibit the binding of Ramos to activated PAEC. The ch2A2 HuG4 and ch3F4 HuG4 produced nearly identical inhibition curves as the murine 3F4 mAb, demonstrating the retention of binding avidity and cell adhesion blocking capabilities in these chimeric antibodies.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 mAbs to inhibit binding of cells expressing both VLA-4 and LFA-1 was next examined using the Jurkat T cell line. Both the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of Jurkat to activated PAEC by up to 90% while the control antibody h5G1.1 HuG4 did not inhibit binding at any concentration (Fig. 4(A)). Similarly, the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of both resting (Fig. 4(B)) and activated (Fig. 4(C)) T cells to activated PAEC, although the

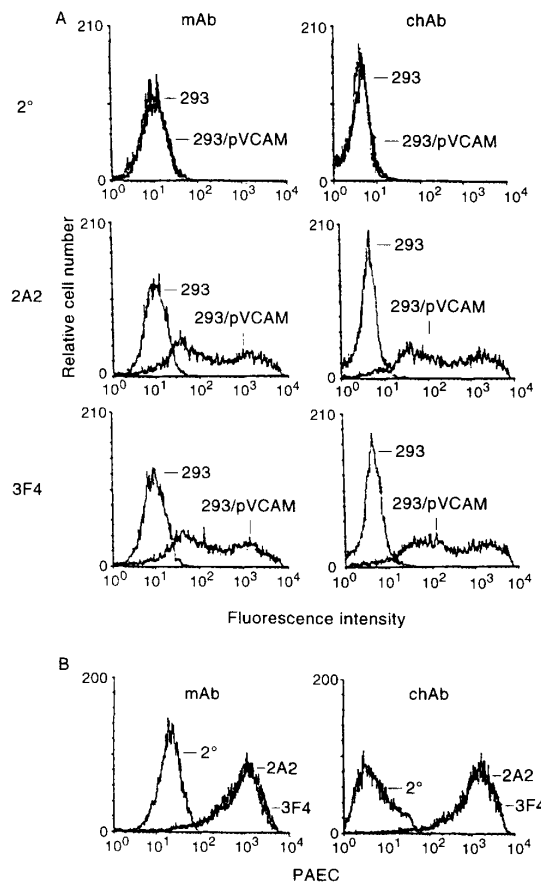


Fig. 2. Binding of 3F4 and 2A2 chimeric antibodies to pVCAM. (A) Chimeric mAbs containing the variable regions of either 2A2 or 3F4 joined to human IgG4 and kappa constant regions were produced in 293-EBNA cells and purified by protein G Sepharose chromatography. Untransfected 293-EBNA cells (293) or 293-EBNA cells expressing pVCAM (293/pVCAM) were incubated with 10 μ g/ml purified murine 2A2 mAb, murine 3F4 mAb, ch2A2 HuG4, or ch3F4 HuG4. Bound antibody was detected using either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-human IgG followed by flow cytometry using a Becton Dickinson FACSsort. Data are displayed as histograms with fluorescence intensity plotted on the x-axis and relative cell number plotted on the y-axis. Background staining by the secondary antibodies is shown in the panels labeled 2°. (B) PAEC were activated by treatment with 1 μ g/ml LPS for approximately 16 hr. Cells were then stained with either the murine 2A2 and 3F4 mAbs (left panel) or the ch2A2 HuG4 and ch3F4 HuG4 antibodies (right panel). Background staining by the secondary antibodies (2°) is indicated.

maximal inhibition achieved was reduced to approximately 60%. Addition of a blocking mAb directed against human VLA-4 also maximally inhibited adhesion by 60% (Fig. 4(C)), confirming the binding inhibition observed was due to blockade of the molecular interaction between human VLA-4 and porcine VCAM.

The potential of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to inhibit binding of natural killer cells and monocytes to activated PAEC was examined using the

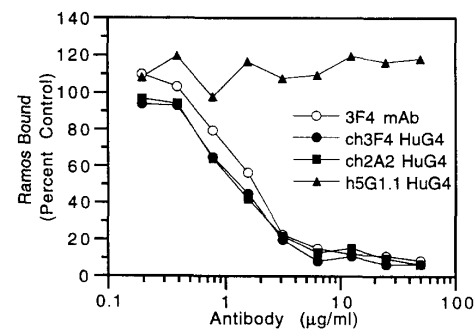


Fig. 3. Binding of Ramos cells to PAEC in the presence of mAbs. On day 0, PAEC were plated at 10 000 cells per well in medium A in 96-well plates. On day 1 the PAEC were activated by adding fresh medium A supplemented with 1 μ g/ml LPS. After 16 to 24 hr incubation, the PAEC were washed and refed RPMI 1640 containing 1% FCS plus the indicated concentrations of the murine 3F4 mAb (3F4 mAb), the chimeric 2A2 and 3F4 mAbs (ch2A2 HuG4 and ch3F4 HuG4) or a humanized negative control antibody (h5G1.1 HuG4). Calcein-labeled Ramos cells were added and allowed to adhere to the PAEC under static conditions for 15 min. After nonadherent cells were removed by washing, the remaining bound cells were lysed with SDS. Calcein fluorescence of the SDS supernatant was measured with a Millipore CytoFluor 2350. In each experiment the binding of Ramos cells to PAEC in the absence of antibody was defined as 100%. Results shown are the mean of two to four experiments, with each value determined in duplicate in each experiment.

cell lines YT (Yodio *et al.*, 1985) and U-937 (Sundstrom and Nilsson, 1976), respectively. Binding of YT cells to activated PAEC was strongly inhibited by concentrations of 10 μ g/ml ch2A2 HuG4 and ch3F4 HuG4 antibodies (Fig. 5(A)). In contrast, the ch2A2 HuG4 and ch3F4 HuG4 antibodies did not inhibit binding of U-937 cells to activated PAEC (Fig. 5(B)). One possible explanation for this failure to inhibit U-937 cell binding could be the presence of elevated levels of VLA-4 on U-937 cells. However, FACS analysis revealed the presence of comparable levels of CD49d and activated CD29 on U-937 cells as on other cell lines such as Jurkat whose adhesion to PAEC was inhibited by the chimeric anti-pVCAM antibodies (Fig. 6). Interestingly, the adhesion of U-937 to PAEC was consistently augmented by approximately 50% in all experiments. This increase was specific for the anti-pVCAM antibodies as no increase in U-937 binding to PAEC was detected in the presence of the control h5G1.1 HuG4 antibody (Fig. 5(B)). This suggested that binding of U-937 to PAEC could be increased through FcR binding to the anti-pVCAM HuG4 antibodies on the PAEC surface. In support of this hypothesis, FACS analysis confirmed the presence of significant levels of Fc γ R1 (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16) on the surface of U-937 cells. In contrast, Jurkat and YT cells had no detectable Fc γ receptors while Ramos cells showed the presence of only low levels of Fc γ RII (Fig. 6).

Binding of the HuG4 chimeric antibodies to U-937 could be directly demonstrated by flow cytometry (Fig.

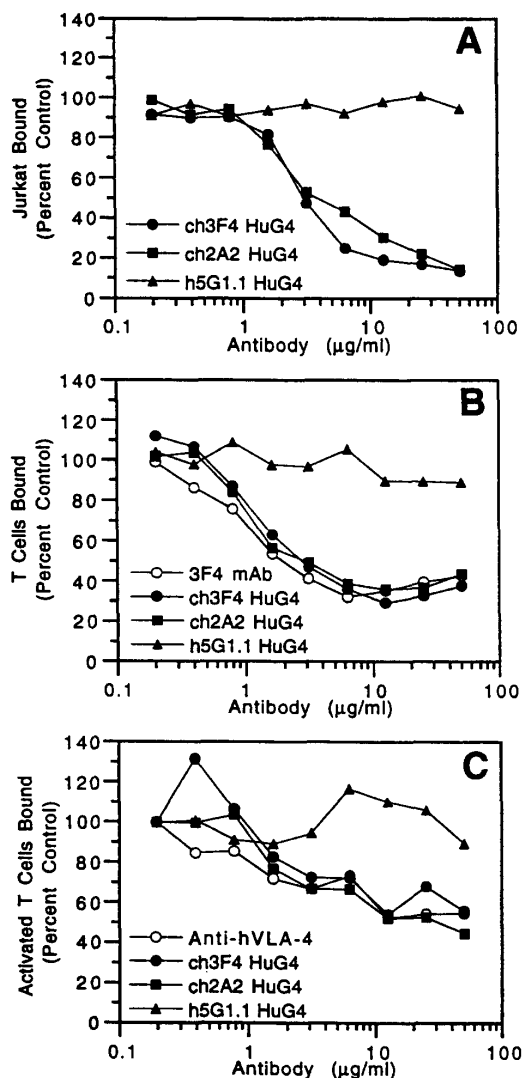


Fig. 4. Binding of Jurkat and human T cells to PAEC in the presence of HuG4 antibodies. Binding experiments were performed as described in Fig. 3 using calcein-labeled Jurkat cells (A) or calcein-labeled purified human T cells (B and C). Human T cells were either assayed immediately after purification (B) or cultured in the presence of 1 µg/ml PHA for 5 days followed by 1 day without PHA stimulation (C) prior to performing the adhesion assay. Results shown are the mean of two to four experiments (A and B) or from an individual experiment (C). Anti-hVLA-4 denotes the blocking murine mAb HP2/1 directed against the $\alpha 4$ subunit of human VLA-4.

7). Both the HuG4 antibodies as well as the anti-human C5 HuG4 antibody bound to U-937 cells. In contrast, 293/pVCAM cells bound only the anti-pVCAM antibodies but not the anti-human C5 HuG4 antibody. No binding of the HuG4 chimeric antibodies could be detected with Ramos, Jurkat, or YT cells. These results suggest that the HuG4 chimeric antibodies bound to U-937 cells through interaction of the HuG4 Fc region with the Fc receptors (FcRs) present on U-937 cells. Finally,

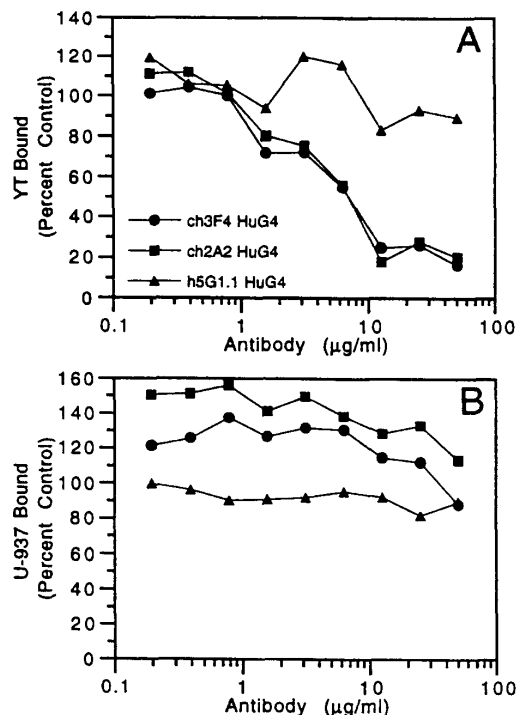


Fig. 5. Binding of YT and U-937 to PAEC in the presence of HuG4 mAbs. Adherence of calcein-labeled YT cells (A) or U-937 cells (B) to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

F(ab')₂ fragments prepared from ch3F4 HuG4 strongly inhibited binding of U-937 cells to PAEC (Fig. 8) whereas F(ab')₂ fragments prepared from h5G1.1 HuG4 did not inhibit the binding. This result demonstrates that, if antibody crosslinking is prevented, blockade of the interaction between human VLA-4 and porcine VCAM can effectively inhibit the adhesion of human monocytes to PAEC.

Inhibition of FcR binding of humanized mAbs with altered constant regions

Although F(ab')₂ fragments proved capable of inhibiting adhesion of U-937 cells, antibody fragments are sub-optimal for chronic treatments, as they have much shorter *in vivo* half-lives than intact antibodies (Covell *et al.*, 1986). The use of intact antibodies with human constant regions as anti-rejection agents is complicated by the capacity of such antibodies to fix C and/or to bind FcRs. Human antibodies of the G4 isotype are devoid of C activity (Tao *et al.*, 1993; Smith *et al.*, 1995). Human antibodies of the G2 isotype do not activate C or bind FcRs (Canfield and Morrison, 1991; Burton and Woof, 1992). Rather than altering the specific residues known to mediate C fixation and FcR binding, we explored the possibility of creating an antibody with a composite human constant region. To create chimeric mAbs which would not activate C or bind to FcRs, we utilized the overall similarity in the human G2 and G4 primary

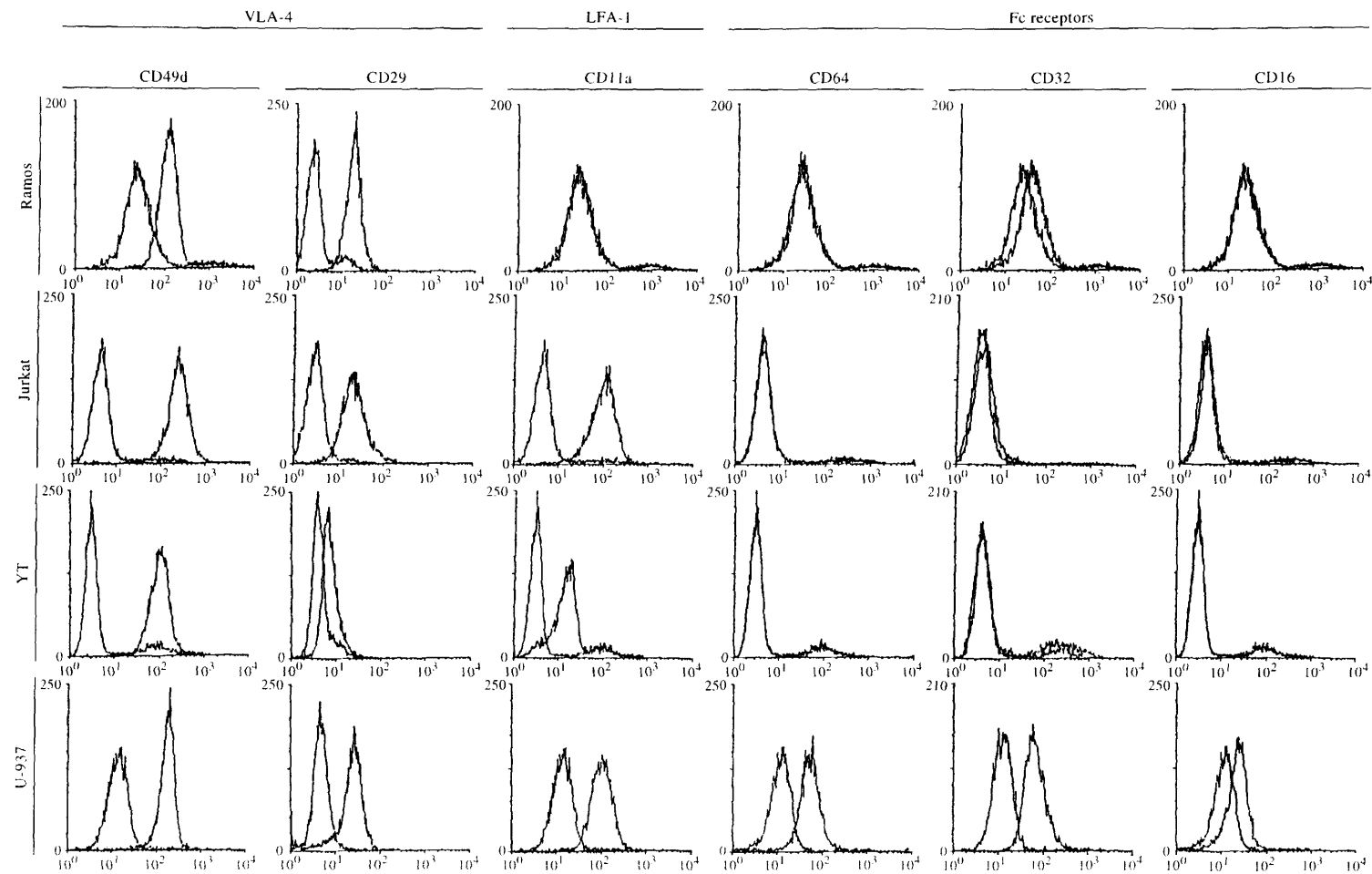


Fig. 6. Expression of VLA-4, LFA-1 and FcRs on the surface of Ramos, Jurkat, YT and U-937 cells. Cells were incubated with $10 \mu\text{g/ml}$ of the following primary antibodies: mouse anti-human CD49d, rat anti-activated human CD29, mouse anti-human CD11a, mouse anti-human CD64 (Fc γ RI), mouse anti-human CD32 (Fc γ RII) or mouse anti-human CD16 (Fc γ RIII). After washing, cells were incubated with either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rat IgG as appropriate. Each panel presents the results obtained in the presence of primary plus secondary antibody or in the presence of secondary antibody alone.

Anti-porcine VCAM antibodies block human leukocyte adhesion

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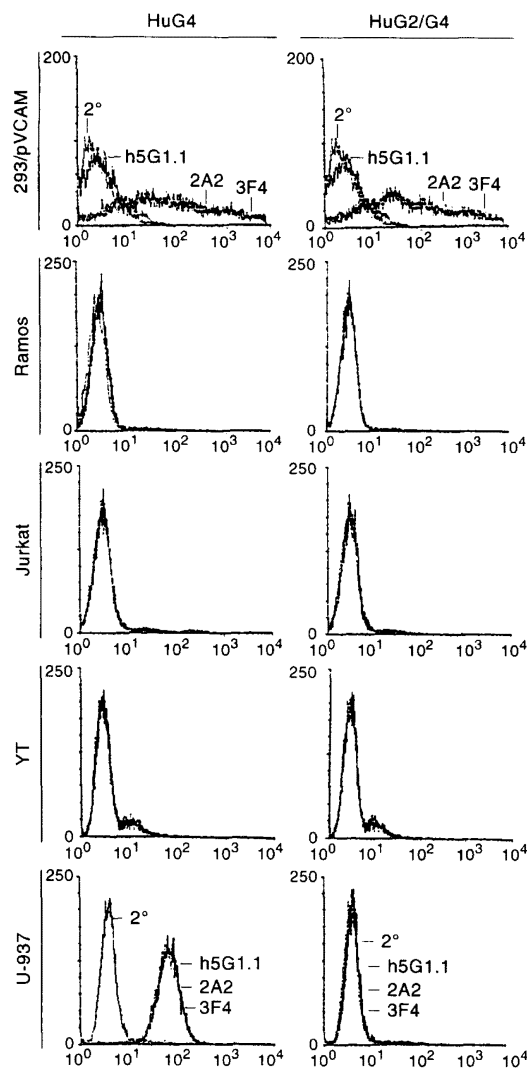


Fig. 7. Binding of chimeric antibodies to 293/pVCAM, Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μ g/ml h5G1.1 HuG4, ch2A2 HuG4, or ch3F4 HuG4 (left panels) or 10 μ g/ml h5G1.1 HuG2/G4, ch2A2 HuG2/G4, or ch3F4 HuG2/G4 (right panels). After washing, cells were incubated with FITC-conjugated goat anti-human IgG except for Ramos cells which were incubated with FITC-conjugated goat anti-human IgG (γ chain specific). Each panel presents the results obtained with the indicated cell line for h5G1.1 HuG4, ch2A2 HuG4 and ch3F4 HuG4 mAbs as well as the secondary antibody alone (2°) control.

sequences to design a HuG2/G4 composite antibody consisting of the first constant region domain and the hinge region from the HuG2 isotype joined to the second and third constant region domains from the HuG4 isotype (Fig. 9). The resulting mAb did not contain the antibody sequences necessary for FcR binding, nor any new epitopes that would likely be immunogenic.

Binding to FcR was examined by using the monocytic cell line U937. FACS analysis demonstrated that the

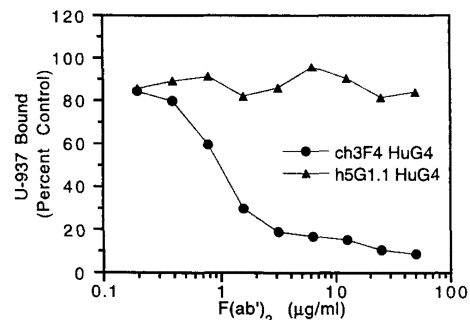


Fig. 8. Binding of U-937 cells to PAEC in the presence of F(ab')₂ fragments. F(ab')₂ fragments were prepared from h5G1.1 HuG4 and ch3F4 HuG4 by digestion with ficin. Undigested antibody and Fc fragments were removed by protein A chromatography. Adherence of calcein-labeled U-937 cells to PAEC in the presence of F(ab')₂ was assayed as described in Fig. 3. Results shown are the mean of five experiments.

resulting ch3F4 HuG2/G4, ch2A2 HuG2/G4 and h5G1.1 HuG2/G4 antibodies did not bind to U-937 cells (Fig. 7). The ch3F4 HuG2/G4 and ch2A2 HuG2/G4 antibodies maintained the ability to bind pVCAM as demonstrated both by binding to 293/pVCAM cells (Fig. 7) and by inhibition of Jurkat and YT binding to activated PAEC (Fig. 10). For both Jurkat and YT cells inhibition of binding with the chimeric HuG2/G4 was equivalent to the HuG4 or original murine anti-pVCAM antibodies. The binding of U-937 cells to PAEC was now potently inhibited by the presence of either ch2A2 HuG2/G4 or ch3F4 HuG2/G4 antibodies. Finally, the chimeric antibodies were examined for the ability to block the adhesion of U-937 cells to purified soluble pVCAM (Fig. 11). Addition of the ch3F4 HuG4 antibody failed to inhibit binding of U-937 cells to pVCAM unless very high concentrations of antibody were added. In contrast the ch3F4 HuG2/G4 antibody potently inhibited the binding of U-937 cells to pVCAM, while the negative control h5G1.1 HuG2/G4 antibody did not block binding. Together, these results demonstrate the anti-pVCAM HuG2/G4 antibodies specifically inhibited the adhesive interaction of human VLA-4 with porcine VCAM and that disruption of this interaction was sufficient to inhibit binding of numerous human cell types to porcine endothelial cells.

DISCUSSION

Current success in allotransplantation has resulted from the development of effective global immunosuppressive therapies that prevent cellular graft rejection. Recent attempts to develop more specific immunosuppressive strategies have focused on inhibiting interactions between integrins and costimulatory molecules on T cells with their ligands on graft endothelial cells and other antigen presenting cells (reviewed in Faustman, 1995; Guinan *et al.*, 1994). The success of allotransplantation has been limited by the resulting shortage of human donor organs. Recent progress in overcoming the initial

HuG2	STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG2/G4	STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG4	STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG2	HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDPKPSNTKVDKTVE
HuG2/G4	HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDPKPSNTKVDKTVE
HuG4	HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDPKPSNTKVDK <u>Q</u> VE
HuG2	²³⁵ RKCCVECPPCAPPV- AGPSVFLF
HuG2/G4	RKCCVECPPCAPPV- AGPSVFLF
HuG4	<u>SKYGP</u> PCP <u>SC</u> PAPEFLGGPSVFLF
HuG2	PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG2/G4	PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG4	PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG2	³³¹ EEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKG
HuG2/G4	EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
HuG4	EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK <u>A</u> KG

Fig. 9. Sequence of the CH1, hinge and CH2 regions of human IgG2 (HuG2), human IgG4 (HuG4), and the chimeric HuG2/G4 construct. Positions where differences occur between the three molecules are double underlined. The amino acid sequence at position 235/236 determines ability to bind to Fcγ receptors (Canfield and Morrison, 1991; Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) while the presence of a serine at position 331 confers the inability to activate C (Tao *et al.*, 1993).

hyperacute rejection of discordant xenografts suggests the potential for use of porcine xenografts as a clinical option to circumvent this chronic shortage. Although there are limited data regarding the fate of discordant xenografts which survive the initial hyperacute rejection phase, xenograft recipients will likely require continued cellular immunosuppression for the prevention of graft rejection. In this light, xenografts offer a unique opportunity for the development of blocking reagents specific for the graft, thereby avoiding global immunosuppression of the recipient.

To investigate the interactions occurring between human leukocytes and porcine endothelial cells, we have cloned the porcine VCAM molecule and created mAbs specific for porcine VCAM that do not bind to human VCAM (Mueller *et al.*, 1995). Two of these antibodies (3F4 and 2A2) are capable of blocking the interaction between human VLA-4 and porcine VCAM. Here we demonstrate that the binding specificity for 2A2 is located within domain 1 of pVCAM while the binding specificity for 3F4 is located within domain 2. This is identical to the results found with human VCAM, where blocking antibodies have also been shown to be directed against either domains 1 or 2 (Osborn *et al.*, 1992; Vonderheide and Springer, 1992).

The use of murine mAbs in humans and primates presents numerous problems including a short antibody half-life and a high degree of immunogenicity (Khazaeli *et al.*, 1994). To optimize the use of the anti-porcine VCAM antibodies in porcine-to-primate xenograft experiments we developed chimeric antibodies using the variable regions cloned from the 2A2 and 3F4 antibodies joined to the constant regions of the human IgG4,κ isotype. Given the extensive activation of C which occurs within xenografts, the human IgG4 isotype was chosen due to its inability to activate C. The resultant chimeric antibodies (ch2A2 HuG4 and ch3F4 HuG4 antibodies) retained

specificity for porcine VCAM and were as potent as the original murine mAbs at blocking adhesion of Ramos cells to activated PAEC by greater than 90% (Fig. 3). In contrast to Ramos, the binding of resting or activated human T cells to activated PAEC was inhibited only approximately 50% by either the chimeric antibodies or the original mAbs (Fig. 4). Activation of human T cells by PAEC can be blocked by antibodies directed against either CD2 or LFA-1 (Rollins *et al.*, 1994b), suggesting either of these molecules could mediate the remaining adhesion by interacting with porcine CD58 or porcine ICAM-1, respectively.

The adhesion of human lymphocytes, monocytes or NK cells to activated HUVEC is dependent on the interaction of both LFA-1 with ICAM-1 and VLA-4 with VCAM (Allavena *et al.*, 1991; Jonjic *et al.*, 1992; Vonderheide and Springer, 1992). For each cell type, blocking the VLA-4/VCAM interaction by itself produces only a small reduction in adhesion, while antibodies which disrupt the LFA-1/ICAM-1 interaction provide a greater, although still partial, inhibition of adhesion. Addition of antibodies blocking both VLA-4/VCAM and LFA-1/ICAM-1 is required to completely block adhesion of these cell types to HUVEC. Surprisingly, binding of LFA-1⁺-VLA-4⁺ Jurkat or YT cells to activated PAEC could be inhibited nearly completely by the ch3F4 HuG4 and ch2A2 HuG4 antibodies. One possible explanation for this could be the lack of interaction between human LFA-1 and porcine ICAM. The conservation of ICAM-1 is relatively low between species, with human and mouse ICAM-1 being only 50% conserved at the amino acid level (reviewed in Carlos and Harlan, 1994), and *in vitro* mouse LFA-1 does not bind to human ICAM-1. Alternatively, human LFA-1 may interact with porcine ICAM-1, but with a reduced affinity. For example, human VLA-4 binds to human VCAM at two independent sites, one within domain 1 and one within

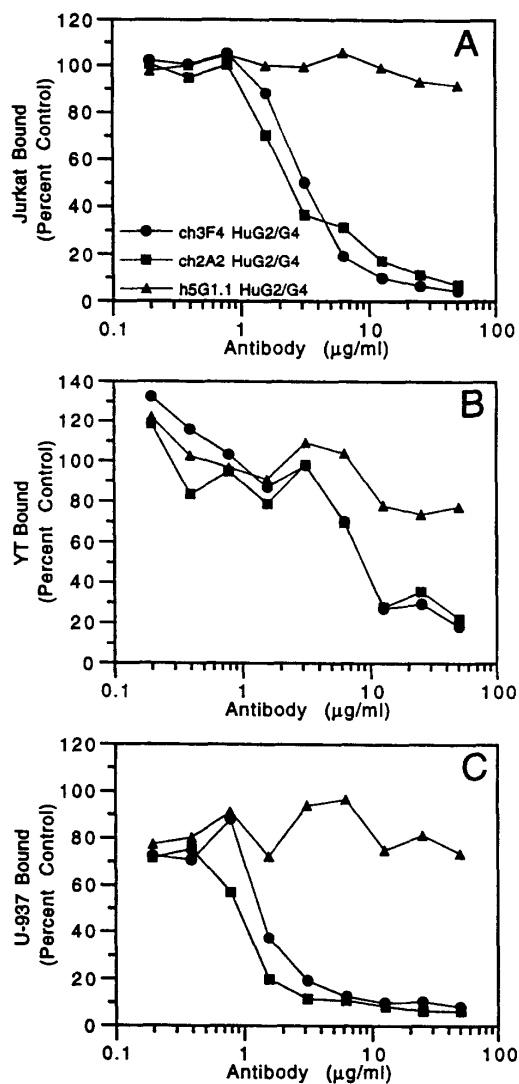


Fig. 10. Binding of Jurkat, YT and U-937 to PAEC in the presence of HuG2/G4 mAbs. Adherence of calcein-labeled Jurkat (A), YT (B) and U-937 (C) cells to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

domain 4 (Osborn *et al.*, 1992; Vonderheide *et al.*, 1994). In contrast, porcine VCAM lacks domain 4, suggesting the interaction between human VLA-4 and porcine VCAM may be weaker than the interaction between human VLA-4 and human VCAM. Reduced ligand affinities could make the binding of human effector cells to porcine endothelial cells more susceptible to disruption of any one of multiple weak interactions. These observations suggest the potential for significant differences in the therapeutic efficacy achieved by inhibiting particular receptor-ligand interactions with xenografts as compared to allografts.

Although the ch3F4 HuG4 and ch2A2HuG4 blocked binding of Jurkat and YT to activated PAEC, both anti-

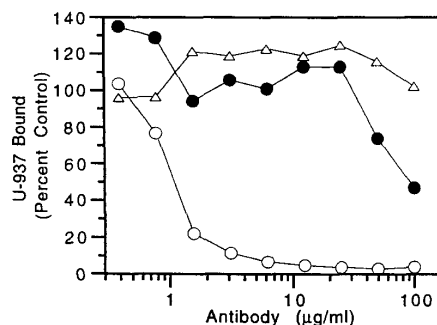


Fig. 11. Binding of U-937 to purified pVCAM. Microtiter plate wells were coated with purified soluble pVCAM. After blocking with FCS, wells received the indicated concentrations of either ch3F4 HuG4 (●), ch3F4 HuG2/G4 (○) or h5G1.1 HuG2/G4 (△) followed by 3×10^5 calcein-labeled U-937 cells. The cells were allowed to adhere at 37°C for 15 min after which non-adherent cells were removed by washing. Adherence of U-937 cells in the absence of competitor antibody was defined as 100%. Results shown are from an individual representative experiment.

bodies failed to block binding of the monocytic U-937 cells to activated PAEC (Fig. 5). The levels of activated $\beta 1$ were comparable on Jurkat and U937 cells indicating an increased level of functional VLA-4 was not the mechanism behind the lack of inhibition of U-937 binding. Additionally, flow cytometry demonstrated the direct binding of the chimeric HuG4 antibodies to U-937 cells (Fig. 7). This binding was not dependent on the antigen specificity of the variable region, suggesting the interaction occurred through the Fc portion of the antibody. Finally, F(ab')₂ prepared from 3F4 HuG4 were able to block the adhesion of U-937 cells to activated PAEC by greater than 80% (Fig. 8). Together these results were consistent with a model in which the chimeric anti-pVCAM HuG4 mAbs crosslinked U-937 cells to activated PAEC by simultaneously binding FcRs and cell surface VCAM present on the PAEC. This is comparable to the situation encountered with the anti-CD3 murine monoclonal antibody (mAb) OKT3, which when first administered to patients produces crosslinking of T cells to FcR bearing cells, resulting in the activation of both cell types (Ceuppens *et al.*, 1985; Kan *et al.*, 1986) with resultant toxicity to the patient.

The interaction of antibodies with Fc γ receptors has been found to be dependent on the sequence of the hinge linker region of the CH2 domain (Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) as well as an additional bend located between two β strands within the CH2 domain (Canfield and Morrison, 1991). In the case of a humanized OKT3 antibody (human IgG4 constant regions), binding to Fc γ receptors has been eliminated by the introduction of amino acid mutations in the hinge linker (Alegre *et al.*, 1992, 1994). In either case the resulting human IgG4 constant region contains amino acid differences which could be immunogenic. Here, we have created chimeric antibodies (designated HuG2/G4) containing the CH1 domain and hinge linker region of

human IgG2 followed by the CH2 and CH3 domains of the human IgG4 constant region. Due to the similarities between these two isotypes at the amino acid level, the resultant antibody contained no novel peptides less than 30 amino acids in length likely to be presented by MHC class II molecules and invoke an immune response. The chimeric HuG2/G4 antibodies no longer bound to U-937 cells. Moreover, the ch2A2 HuG2/G4 and ch3F4 HuG2/G4 antibodies blocked the binding of Jurkat and YT cells to activated PAEC as potently as the chimeric HuG4 antibodies, demonstrating the alteration of the Fc region did not reduce the antibody avidities. Moreover, the chimeric anti-pVCAM HuG2/G4 recombinant antibodies nearly completely blocked the binding of U-937 cells to activated PAEC and to purified pVCAM. Finally, as predicted from the low affinity of IgG2 and IgG4 for human C1q, our preliminary results indicate that the HuG4 and HuG2/G4 recombinant antibodies show little or no C1q binding (data not shown).

In summary, we have developed humanized mAbs specific for porcine VCAM which potently block the adhesion of multiple human inflammatory cell types to porcine endothelial cells. The lack of binding of these antibodies to human VCAM leaves intact the interaction of human leukocytes with human endothelial cells. The adhesion-blocking properties of these antibodies will allow delineation of the role of VLA-4/VCAM interactions in cellular mediated rejection of porcine-to-primate xenografts. Additionally, the HuG2/G4 antibody design should prove useful in humanization of other antibodies intended for human use where elimination of FcR binding and C activation may be desirable.

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