Title

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A Homing Receptor–IgG Chimera as a Probe for Adhesive Ligands of Lymph Node High Endothelial Venules

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Abstract. The binding of lymphocytes to high endothelial venules (HEV) within peripheral lymph nodes (pln) is thought to be mediated by a lectinlike adhesion molecule termed the pln homing receptor (pln HR). The cloning and sequencing of cDNAs encoding both murine and human pln HR revealed that these adhesion molecules contain protein motifs that are homologous to C-type or calcium dependent lectin domains as well as to epidermal growth factor (egf) and complement-regulatory protein domains. We have produced a novel, antibody-like form of the murine HR by joining the extracellular region of the receptor to a human IgG heavy chain. This antibody-like molecule is capable of recognizing carbohydrates, blocking the binding of lymphocytes to pln HEV, and serving as a histochemical reagent for the staining of pln HEV. This murine HR–IgG chimera should prove useful in analyzing the distribution of the HR ligand(s) in normal as well as in inflammatory states.

LYMPHOCYTES bind to and extravasate through specialized high endothelial venules (HEV)[1] that are found in secondary lymphoid organs such as peripheral lymph nodes (pln) and Peyer's patches (pp) (8, 15, 42, 52, 53). The ability of lymphocytes to migrate selectively to one lymphoid organ as compared to others has been termed homing. There is evidence that a set of diverse adhesion receptors on lymphocytes ("homing receptors"; HR) may be involved in the ability of lymphocytes to home to different anatomical regions such as pln, pp, and lung (8, 10, 16). While the biochemical identities of the lymphocyte homing receptors and their cognate endothelial cell ligands are only beginning to be understood, it seems likely that these systems are intimately involved in not only the trafficking of lymphocytes to the organized lymphoid organs of the body but also in the extravasation of various leukocytes at sites of inflammation (22, 24, 25, 29, 33, 37).

The best-characterized adhesion molecule involved in lymphocyte homing is the 90-kD glycoprotein (gp90^{MEL}) defined on mouse lymphocytes by the MEL-14 mAb. Closely related proteins of somewhat greater molecular mass are present on the surfaces of neutrophils and monocytes (29). Initial studies demonstrated that the MEL-14 mAb blocks the attachment of lymphocytes to pln HEV both in vitro and in vivo (15, 32) but has no effect on their binding to pp HEV. In addition, the presence of the MEL-14 defined epitope on various lymphocyte and lymphoma populations correlates

with the ability of the cells to bind to pln HEV (15, 34). Recently, it was found that purified gp90^{MEL}, when reacted with sections of lymphoid organs, blocks the binding of lymphocytes to pln HEV but not to pp HEV, suggesting that the glycoprotein forms a direct bridge from the lymphocyte to endothelial ligands in an organ-restricted manner (17). Thus, the accumulated evidence warrants the designation peripheral lymph node (pln) HR for gp90^{MEL}. Evidence developed in parallel to these studies has revealed that the murine pln HR (i.e., gp90^{MEL}) and its human homologue function as calcium-dependent, lectinlike receptors. In rat, mouse, and human systems, lymphocyte attachment to pln HEV, a calcium-dependent cellular interaction (7, 51), is competitively inhibited by specific carbohydrates such as D-mannose-6-phosphate (M6P), the M6P-rich polysaccharide called PPME, and the sulfated fucose-rich polysaccharide known as fucoidin (43, 44, 46, 54). Studies examining the interaction of PPME with the lymphocyte surface in the mouse pointed to the mHR (i.e., gp90^{MEL}) as the relevant lectinlike receptor (54, 55). In agreement with this prediction, cDNAs for gp90^{MEL} encode a transmembrane protein containing three adjacent extracellular protein motifs: a calcium-dependent ("C-type") lectinlike domain (11) at the amino terminus, an epidermal growth factor (egf)–like domain and two identical repeats related to those found in a number of complement-regulatory proteins (28, 39). Confirmation of the lectin character of gp90^{MEL} has come from the direct demonstration that the isolated native protein exhibits calcium-dependent, carbohydrate-binding activity in an ELISA assay (21). The importance of the lectin domain in HEV binding was recently supported by the demonstration that the MEL-14–

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1. *Abbreviations used in this paper*: egf, epidermal growth factor; HEV, high endothelial venules; HR, homing receptor; M6P, mannose-6-phosphate; pln, peripheral lymph nodes; pp, Peyer's patches.

defined epitope maps to the N-terminal region of the lectin motif (5) and by the finding that the adhesion-blocking activity of isolated gp90^{MEL} on pln HEV can be prevented by co-incubation of the molecule with M6P and related sugars (17). These findings strongly argue that that the lectin domain of murine pln HR recognizes and binds to a carbohydrate-based ligand that is specifically expressed on the pln HEV. In support of this model, the susceptibility of adhesive ligands on pln HEV to sialidase treatment has provided preliminary evidence for the existence of essential carbohydrate moieties on pln HEV (35, 36).

Consistent with data on cross-species recognition of pln HEV by human and murine lymphocytes (44, 46) human cells express a closely related homologue of the murine pln HR that probably functions in an analogous manner (4, 38, 49). Furthermore, at least two other adhesion molecules, the endothelial leukocyte adhesion molecule (ELAM-1) (2) and the platelet membrane granule protein, GMP 140/PADGEM (3, 23, 31), show the same arrangement of protein motifs as gp90^{MEL} and its human homologue, suggesting that this family of lectin egf complement-cell adhesion molecules or LEC-CAMs (42), may be widespread in the vascular system.

Reagents that are able to specifically recognize the HR ligand on pln HEV might prove useful in analyzing the role of this adhesion system at sites of leukocyte extravasation (e.g., inflammatory foci) outside of organized lymphoid tissues. In addition, such reagents might also act as effective antiinflammatory drugs, if they are able to block leukocyte–endothelial interactions. One method for the derivation of such reagents is to produce mAbs specific for HR ligands found on the surface of the endothelium of HEV (47, 48). As a novel approach, we have developed a chimeric protein containing the murine HR and the hinge and constant regions of the human immunoglobulin heavy chains (9), thus converting the pln HR into a monoclonal antibody-like molecule specific for the cognate adhesive ligand(s) expressed on pln HEV. In the present study, we demonstrate that this chimera exhibits the lectin properties and the adhesion-blocking activity of the native receptor. Additionally, we establish the utility of this protein as a histochemical reagent for staining of pln HEV. The results suggests that the receptor–IgG chimera may prove exceptionally useful in both the isolation of the HEV ligand(s) as well as in the examination of the role of this adhesion system in various inflammatory states.

Materials and Methods

Construction, Analysis, and Purification of Truncated mHR–IgG Chimera

Starting with a previously described mHR-PRK5 expression plasmid (27, 28) and a cDNA encoding a human heavy chain IgG (9), we inserted an 1,100-bp Hind III fragment encoding the CH1-CH3 regions of the human IgG1 constant region 3 prime of the poly A addition site of the mHR cDNA. This plasmid was converted to single-stranded template by using an m13 origin of replication and the K07 helper phage, after which regions between the hinge and the second complement binding repeat (NH2 terminus to the putative trans-membrane region) were looped out with 48-mer oligonucleotides by in vitro mutagenesis (56). The resultant mutants were screened with ^{32}P-labeled 21-mer oligonucleotides spanning the deletion junctions, and the isolated mutants were sequenced using supercoil sequencing. Correct mutants were tested for expression by transfection onto human kidney 293 cells using previously described methods (27). [^{35}S]Methionine and cysteine supernatants were analyzed by immunoprecipitation with protein

A-Sepharose beads in the absence of added antibodies. The precipitated proteins were analyzed on 7.5% polyacrylamide-SDS gels either with (2-mercaptoethanol) or without reduction. Plasmids that resulted in correctly expressed chimeras were introduced into 293 cells by transfection in the presence of selective plasmids encoding resistance to G418 as well as dihydrofolate reductase. Clones were selected in G418, and the incorporated plasmids were amplified in the presence of methotrexate (26). Permanent cell lines expressing high levels of mHR-IgG were grown to large scale in T-flasks or roller bottles, and the cell supernatants were clarified by centrifugation and filtration. The resultant supernatants were concentrated by filtration (Amicon Corp., Danvers, MA) and passed over protein A-Sepharose columns, washed with PBS, and eluted with 0.1 M acetic acid, and 0.15 M NaCl (pH 3.5). The eluted material was immediately neutralized with 3 M Tris (pH 9), and analyzed by SDS gel electrophoresis. Alternatively, concentrated conditioned medium supernatants containing mHR-IgG chimeras were employed. Purified mHR-IgG chimera was quantified with an ELISA format in which an anti–human IgG1-Fc specific mouse mAb, coated onto microtiter wells, was used to capture the chimera protein. Unknown samples and highly purified human CD4-IgG1 immunoadhesin (9) standard (the kind gift of S. Chamow, Department of Process Sciences, Genentech, Inc.), were incubated with antibody-coated plates, after which the plates were washed, and the bound material was reacted with HRP-conjugated goat anti–human IgG1, followed by further washes and addition of substrate. This quantitative assay permitted the measurement of nanogram quantities of mHR-IgG chimeras.

Analysis of mHR-IgG Chimera PPME Reactivity by ELISA

The ability of the mHR-IgG chimera to recognize the yeast polyphosphomannan (PPME), was analyzed in an ELISA format as described (19). Briefly, purified mHR-IgG chimera in Dulbecco's PBS was coated onto Immulon-2 microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4°C. Nonspecific sites were blocked with PBS-BSA at 22°C, after which the bound antigens were reacted with PPME at 1 μg/ml. PPME was prepared from a core mannan preparation kindly provided by Dr. M. Slodki (U.S. Department of Agriculture, Northern Regional Center, Peoria, IL). Various additives, including MEL-14 mAb (10 μg/ml, final concentration), carbohydrates, or EGTA (10 mM, final concentration), were added before PPME incubation in assays examining inhibition. After 1 h at 4°C, the plates were washed and incubated with a rabbit polyclonal antibody directed against PPME for 1 h at 22°C. Plates were washed and incubated with biotinylated goat anti–rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) for 1 h at 22°C followed by incubation with Vector ABC-alkaline phosphatase for 30 min. The plates were developed after addition of substrate and read with a microplate reader. All determinations were carried out at least in triplicate. The background level, defined as the signal in the absence of PPME was subtracted from all values to yield the specific signal. Chondroitin sulfate A, heparin (porcine intestinal mucosa) and brain sulfatide were obtained from Sigma Chemical Co. (St. Louis, MO), and fucoidin came from K&K Laboratories (Plainview, NY).

Cell Blocking Assays with mHR-IgG Chimera

The Stamper-Woodruff cell blocking assay (51) was performed with cryostat-cut sections of mouse pln and pp as previously described (17). The tissue sections were preexposed to buffer alone, purified mHR-IgG chimera, or gp90^{MEL}, which was purified and equilibrated in low detergent buffer as previously described (28). After the sections were washed, the lymphocyte adherence assay was carried out with mesenteric lymph node lymphocytes added at 1 × 10^7 cells/ml (17). Adherence was quantified by digital morphometry as the number of lymphocytes bound per unit area of HEV and referenced as a percentage of control binding in the absence of inhibitors.

Immunohistochemical Analysis of mHR-IgG Chimera

The mHR-IgG chimera was employed for histochemical staining based on standard procedures with mAbs. Briefly, 10-μM tissue sections were cut in a cryostat and fixed with 0.5-1.0% paraformaldehyde in 0.1 M cacodylate (pH 7.3) for 20 min at 4°C, followed by immersion in 100% methanol with 0.3% H$_2$O$_2$ for 20 min at 4°C to eliminate endogenous peroxidase. The sections were washed in Dulbecco's PBS and incubated for 60 min at 4°C with the indicated amounts of mHR-IgG chimera (either protein A purified or as concentrated cell supernatants from culture medium) diluted in 5%
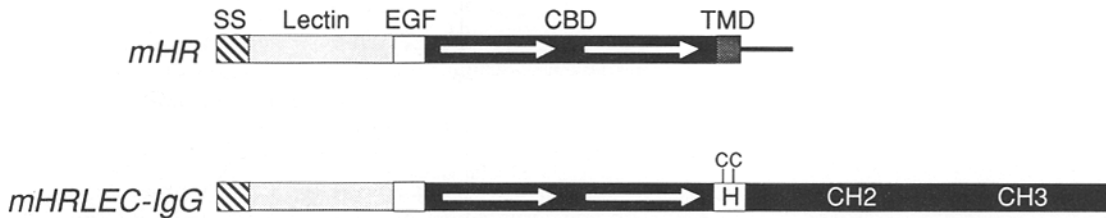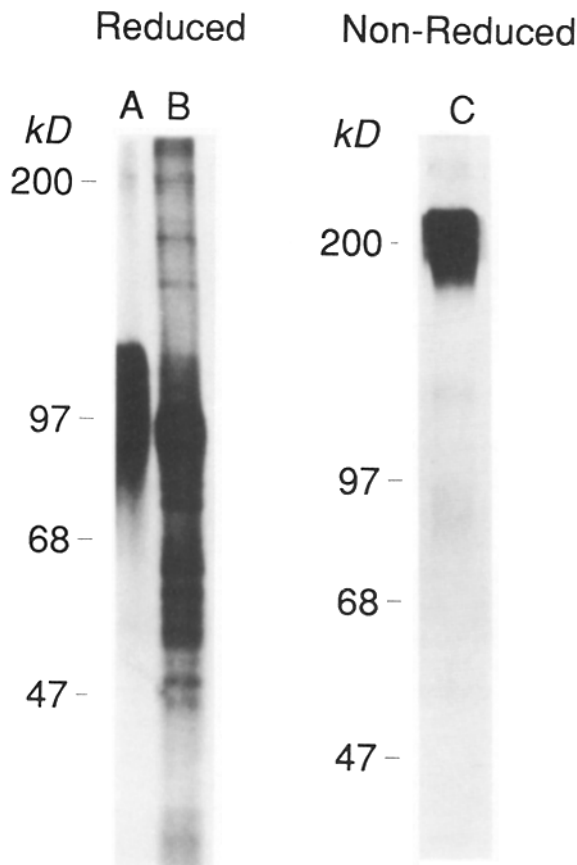
A

Murine PLN Homing Receptor IgG Chimera

B

Figure 1. Construction and expression of a mHR-IgG chimera containing the lectin-egf complement binding motifs. (*A*) The protein domains of the murine homing receptor (mHR) are shown: NH_2-terminal signal sequence (*SS*), lectin, egf, and duplicated complement binding domains (*CBD*), transmembrane anchor domain (*TMD*), and a short cytoplasmic sequence. The truncated mHR-IgG chimera that contains the lectin, egf, and two complement binding motifs is shown below. This truncated protein is joined to a human heavy chain gamma-1 region immediately NH_2-terminal to the hinge domain (*H*) such that this chimera contains the two cysteine residues (*C*) of the hinge responsible for immunoglobulin dimerization as well as the CH2 and CH3 constant regions. (*B*) 293 cells were transiently transfected with an expression plasmid containing mHRLEC-IgG chimera, labeled with [^{35}S]cysteine and methionine, and the whole cell lysates (extracted in 1% NP-40/0.1% SDS) and secreted materials were precipitated with protein A-Sepharose beads in the absence of added antibody after which the resultant precipitates were eluted from the beads with SDS in the presence or absence of the reducing agent 2-mercaptoethanol and electrophoresed on SDS–polyacrylamide gels and subjected to autoradiography. Reduced proteins: lane *A*, secreted material; lane *B*, whole cell lysate. Nonreduced protein: lane *C*, secreted material. For brevity, the mHRLEC-IgG chimera is referred to as mHR-IgG chimera in the text of the paper.

normal mouse serum in PBS. The sections were then washed and incubated with either biotinylated goat anti–human Fc-specific antibody (Vector Laboratories, Inc.) or affinity-purified biotinylated goat anti–human IgG (Zymed Laboratories, San Francisco, CA) in PBS containing 5% normal mouse serum for 30 min at 22°C. Sections were washed and incubated with AEC peroxidase substrate (Biomeda, Foster City, CA) for 5–10 min. Finally, the sections were counterstained with aqueous hematoxylin (Biomeda) and viewed with a Nikon Optophot.

PPME binding of mHR-IgG chimera
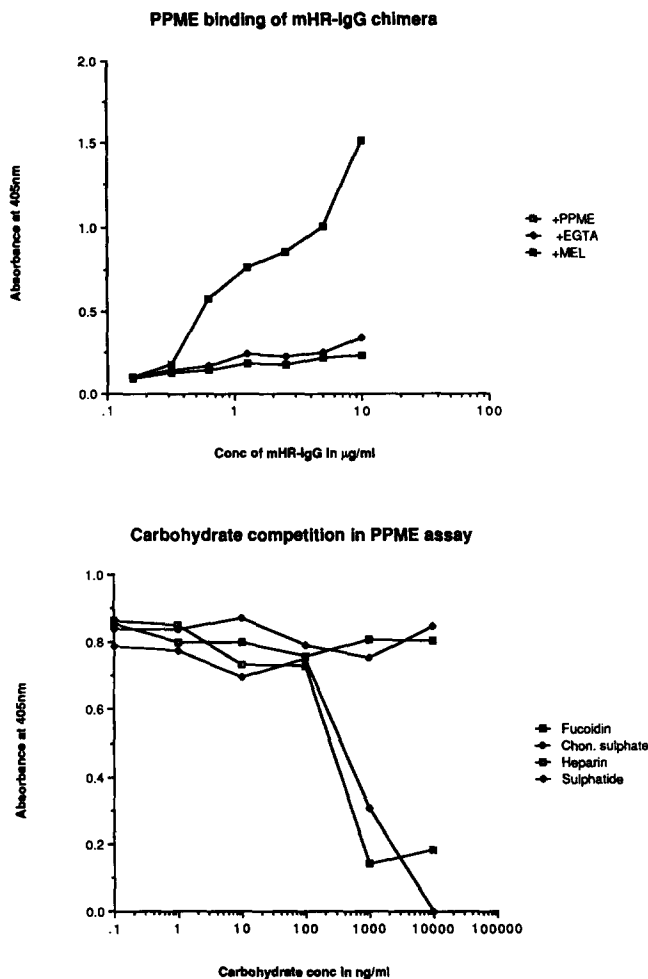
Carbohydrate competition in PPME assay

Figure 2. Binding of mHR-IgG to PPME. Purified mHR-IgG was quantified using an ELISA assay as described in Materials and Methods. The purified chimera was coated onto microtiter wells and reacted with PPME, after which the bound PPME was detected with a polyclonal antibody in conjunction with alkaline phosphatase-ABC reagents (21). Inhibition with MEL-14 mAb was performed by preincubating mHR-IgG containing wells with the mAb (10 µg/ml) before addition of PPME, while the calcium dependence of the HR–carbohydrate interaction was demonstrated by inclusion of 10 mM EGTA during the binding reaction. Inhibition of PPME binding with various carbohydrates was examined as described in Materials and Methods. 50 µliter aliquots of chimera (1 µg/ml) were used to coat the microtiter wells for the carbohydrate inhibition studies. (*Top*) Binding of PPME to increasing quantities of mHR-IgG and inhibition of mHR-IgG-PPME binding with MEL-14 mAb and EGTA. In the absence of added PPME, the curve was identical to the +PPME+EGTA and the +PPME+MEL-14 mAb conditions. (*Bottom*) Inhibition of mHR-IgG-PPME binding with carbohydrates.

Results

Production of a Murine HR-Human IgG Chimera

We chose to produce a receptor-immunoglobulin constant region chimera for several reasons. First, the production of a chimeric IgG-containing molecule would allow us to produce, purify, and quantify the amount of the chimera using relatively simple, available assays. Second, the ability of this

molecule to dimerize might be expected to add to the avidity of the interaction between the receptor and its ligand on the endothelial surface. Finally, we felt that inclusion of the IgG constant region would permit the use of the chimeric protein in histochemical studies employing readily accessible reagents.

A mHR-IgG chimera was produced by using a previously characterized human heavy chain IgG1 constant region cassette (9). The choice of junctional sites between the mHR and human IgG sequences was guided by work with human CD4-IgG chimeras that demonstrated that the joining of the molecules near the hinge region resulted in chimeric molecules that were both efficiently synthesized and dimerized in the absence of any light chain production (9). In addition, the use of the human IgG1 constant region would eliminate cross-reactivity with endogenous murine IgGs in the immunohistochemical staining of mouse lymphoid organs. Fig. 1 *A* illustrates the chimeric molecule containing the lectin, egf, and complement-binding domains (mHRLEC-IgG) and the human IgG1 heavy chain region that was produced by in vitro deletion mutagenesis (56). The construct was transfected into human kidney cells (27), and the synthesized protein (referred to as mHR-IgG chimera) was recovered by affinity chromatography with protein A-Sepharose beads. As shown in Fig. 1 *B*, the chimera was efficiently synthesized and secreted in the transient transfection assays. The reactivity of the chimera with protein A-Sepharose demonstrated that the constant region domain was normally folded. Fig. 1 *B* also shows that this molecule dimerized under nonreducing conditions, indicating that the hinge region was fully functional in this chimera. Finally, the protein A reactivity also allowed for the purification of this chimera to near homogeneity on protein A-Sepharose columns. Thus, this molecule represents an antibody-like entity whose "variable" domain may be said to be derived from the mHR while the constant domain is derived from the human IgG$_1$ heavy chain.

Analysis of mHR-IgG Chimera for PPME Binding

Previous studies demonstrated that gp90[MEL], either as a cell surface-associated molecule (44, 46, 54) or as an isolated molecule (17), is able to bind to the M6P-rich polysaccharide, PPME. In both cases, MEL-14 mAb inhibits the interaction as does EGTA, a chelator of calcium ions. We thus analyzed the ability of the mHR-IgG chimera to interact with PPME in an ELISA binding assay (21). As described in experimental procedures, this assay employs a microtiter format in which the chimera was bound to plastic and allowed to react with PPME after which the amount of bound PPME was detected with a polyclonal antibody against PPME. Since the mHR-IgG chimera contained the Staphylococcal protein A-binding human IgG1 constant region, the relative amounts of chimera contained in each well could be easily measured by the degree of binding to a protein A-peroxidase conjugate.

As shown in Fig. 2, the binding of PPME to the mHR-IgG chimera was a direct function of increasing IgG chimera levels. The binding was quantitatively similar to that found with comparable quantities of gp90[MEL] isolated from spleen lymphocytes (21). The signal was inhibited by the MEL-14 mAb and was calcium dependent as inferred from the complete
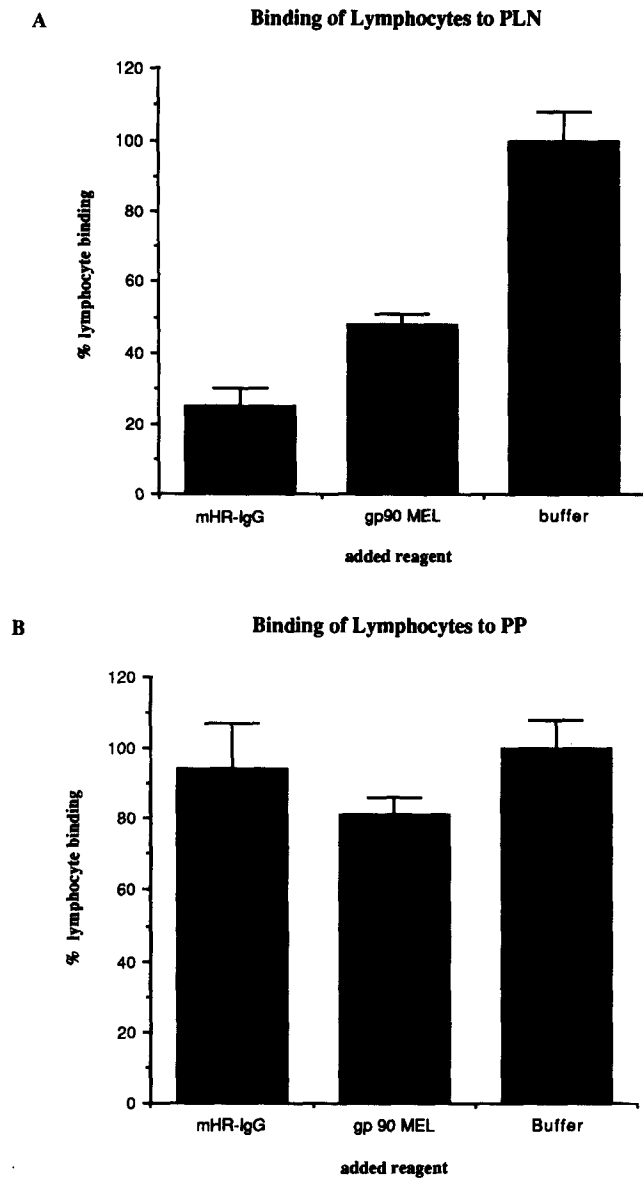
A

Binding of Lymphocytes to PLN

120 —
100 —
80 —
60 —
40 —
20 —
0 —

% lymphocyte binding

mHR-IgG   gp90 MEL   buffer

added reagent

B

Binding of Lymphocytes to PP

120 —
100 —
80 —
60 —
40 —
20 —
0 —

% lymphocyte binding

mHR-IgG   gp 90 MEL   Buffer

added reagent

Figure 3. Inhibition of lymphocyte attachment to pln HEV by mHR-IgG chimera and by gp90^{MEL}. Cryostat sections of pln and pp were pretreated with buffer alone, purified mHR-IgG chimera or spleen-derived gp90^{MEL}, both at 1 μg/section. After the sections were washed, lymphocytes, that had been preincubated with 100 μg/ml aggregated human IgG to reduce Fc receptor interactions, were added to the sections. Binding to HEV is expressed as a percentage of the value in the buffer control condition. Error bars indicate SEMs based on four or more replicate sections for each treatment. Samples were coded and evaluated blindly and at least 15 segments of HEV were counted per section. (*A*) Inhibition of binding of lymphocytes to pln HEV. (*B*) Lack of inhibition of binding of lymphocytes to pp HEV.

elimination of specific binding in the presence of EGTA (Fig. 2 *A*). Thus, as with splenocyte-derived gp90^{MEL}, we conclude that the C-type lectin domain of the chimera was responsible for the interaction with PPME.

Previous work has demonstrated that a variety of carbohydrates in addition to PPME were recognized by the spleen derived mHR (21, 54). These active glycoconjugates included fucoidin and brain-derived sulfatide. The ability of

these carbohydrates to inhibit the interaction between the mHR-IgG chimera and PPME was examined to compare the specificity of this molecule to that of the spleen-derived receptor (21). As shown in Fig. 2 *B*, fucoidin and sulfatide were both effective in inhibiting PPME binding to mHR-IgG, indicating that carbohydrate specificity was retained in the recombinant chimera. The lack of inhibition by two other charged carbohydrates, chondroitin sulfate and heparin, demonstrated that the inhibition resulted from specific carbohydrate recognition and was not attributable to nonspecific charge interference (21).

Analysis of the mHR-IgG Chimera in Cell Binding Assays

While the above findings established that the mHR-IgG chimera recognized specific carbohydrates, they did not address the ability of this chimera to recognize ligands on the endothelium of pln HEV. Since previous work (17) has shown that gp90^{MEL} isolated from splenocytes by detergent extraction is able to inhibit the binding of lymphocytes to pln sections in the in vitro adherence assay (41), we examined the activity of the IgG chimera in this cell binding assay. As shown in Fig. 3 *A*, the mHR-IgG chimera ($\sim$1 μg/section), when prereacted with pln tissue sections, inhibited lymphocyte attachment to HEV by $\sim$75%. Spleen-derived gp90^{MEL} ($\sim$1 μg/section) was also active in the same assay. The lack of complete inhibition of lymphocyte binding by either the mHR-IgG chimera or the spleen-derived material may have been due to either insufficient quantities of blocking proteins or of accessory adhesion molecules such as the CD11/18 integrin system (13). Consistent with the previous findings with gp90^{MEL} (17), the mHR-IgG chimera did not affect the binding of lymphocytes to pp HEV (Fig. 3 *B*). These results indicated that the mHR-IgG chimera was able to effectively compete with lymphocytes for binding to HR ligands on pln but not on pp HEV.

The Use of the mHR-IgG Chimera as a Histochemical Reagent

The blocking of cell binding by the mHR-IgG chimera in the in vitro adherence assay implied that this molecule was capable of a direct interaction with a ligand(s) on the pln HEV. Since this chimera contained the human IgG constant region, we felt that it could be used as a histochemical reagent just as adhesion-blocking mAbs have been employed for the detection of potential ligands on HEV (47, 48). In the case of the mHR-IgG chimera, however, the actual HEV-ligand to which the mHR binds would be directly identified, and the issue of identity vs. proximity of the epitope-bearing molecule and the actual adhesive ligand would be avoided. The result would be a highly specific assay for the presence of the HR ligand not only on pln HEV but also at other endothelial sites where leukocytes use the mHR or related receptors for adherence and extravasation.

Fig. 4 demonstrates that mHR-IgG chimera, used in conjunction with a biotinylated goat anti-human IgG and the HRP-ABC reagent, stained pln HEV. The staining was always confined to the high walled endothelial cells of the HEV. Other structures in the lymph node, including lymphocytes and non-HEV blood vessels were negative. In many instances, the staining appeared to be concentrated on the
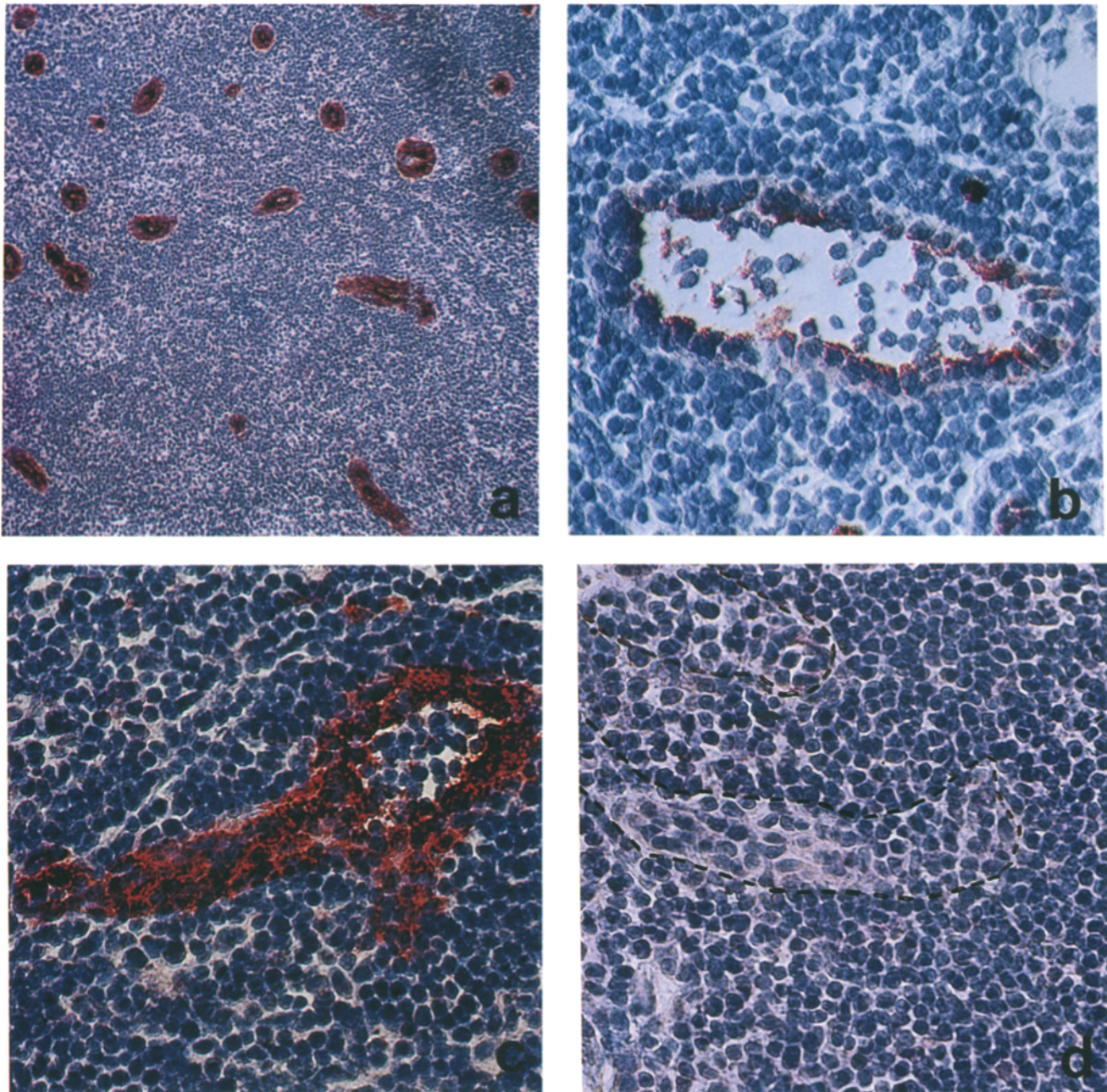
Figure 4. Histochemical staining of HEV with the mHR–IgG chimera. Cryostat-cut sections of a mouse pln and a pp were reacted with the chimera (3 μg of chimera/section, concentrated culture medium supernatant) and processed for HRP histochemistry as described in Materials and Methods. (*a*) Staining of pln, magnification of 140. All the HEV are stained. (*b*) A single pln HEV, magnification at 560. Apical staining of the endothelial cells is apparent. Since the tissue section was paraformaldehyde-fixed before exposure to the chimera, the apical staining probably does not result from a homing receptor-induced redistribution of ligand. The occasional positive cells among the lymphocytes in the node parenchyma are due to nonspecific staining. Equivalent staining is seen in second-stage controls (no chimera added). The staining associated with HEV is absent when chimera is not added. Nonspecific staining is especially prevalent in medullary and subcapsular sinuses of nodes. (*c*) A pln HEV magnification at 560. Staining is seen across the entire thickness of the HEV but is accentuated at the luminal face of the HEV seen at the right. Unstained lymphocytes are present in the lumen of the HEV and in the parenchyma of the node. (*d*) Two pp HEV, magnification at 560. The HEV, with their basement membranes delineated by dashed lines, are largely unstained. Slight staining is present in the upper HEV. The tissue sections in *c* and *d* were stained in parallel under identical conditions.

luminal surface of the specialized endothelial cells as compared to the basolateral surfaces (Fig. 4 *B*). The staining of HEV was blocked by co-incubation of the chimera with the MEL-14 mAb (Fig. 5 *b*) or with EGTA (Fig. 5 *c*), indicating that the binding of mHR-IgG to pln HEV mimicked the inter-

action between lymphocytes and the HEV. In concordance with the ELISA assay presented above, staining of pln HEV was inhibited by fucoidin (Fig. 5 *d*) but not by chondroitin sulfate (not shown). Thus, as previously shown indirectly for gp90[MEL] (17), the carbohydrate-binding activity of mHR-
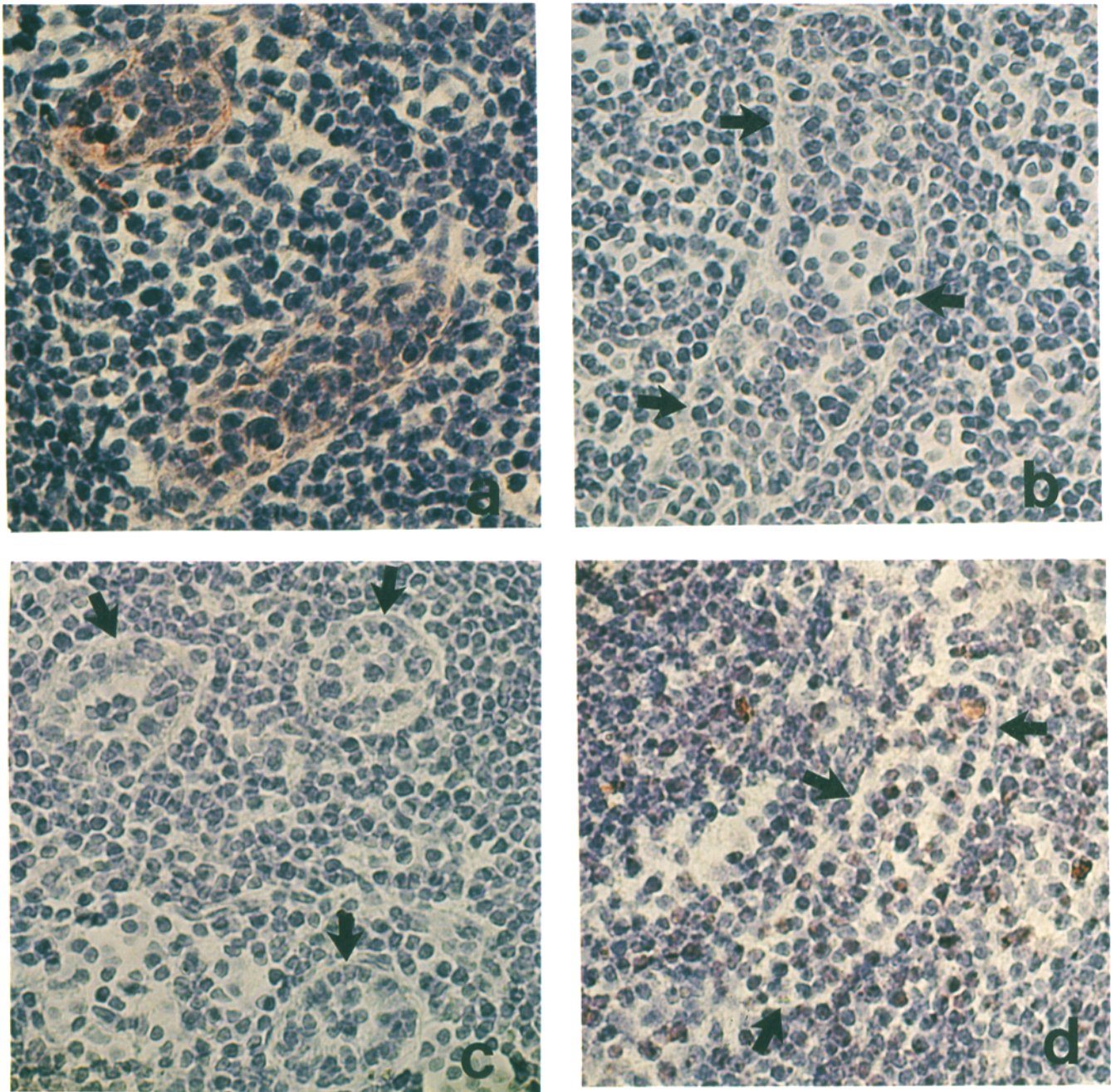
Figure 5. Inhibition of mHR-IgG chimera staining of pln HEV. Sections of pln were stained with 1 μg/section of purified chimera under four conditions. (*a*) Control, no inhibitors present. Two stained HEV are evident, the top one in cross-section and the bottom one in longitudinal section. The staining is not as intense as that in Fig. 4. The parameters responsible for this variability in control staining are not understood. (*b*) In the presence of MEL-14 mAb, 5 μg/section. A single HEV (*longitudinal section*) with its basement membrane delineated by arrows is not stained. (*c*) In the presence of EGTA (10 mM, final concentration). Three HEV indicated by arrows (*cross-sectional profiles*) are not stained. (*d*) In the presence of fucoidin at 10 μg/section. An HEV in longitudinal profile with its basement membrane delineated by arrows is not stained. Magnification for all micrographs is 560.

IgG was essential for its interaction with pln HEV-ligands. Consistent with the failure of mHR-IgG or gp90[MEL] to block the adhesive sites on pp HEV and the known involvement of a distinct adhesive system in this interaction (20), the chimera produced very faint or undetectable staining of the HEV in this lymphoid organ (Fig. 4 *b*). This degree of differential staining was observed in over ten independent experiments. However, in rare cases, moderate staining of pp HEV was seen, although the intensity was always significantly less than observed for pln HEV processed in parallel (not shown).

Discussion

The results reported in this paper describe the use of a soluble, recombinant form of a HR as an antibody-like entity. By

cell adhesion experiments and histochemical staining, we have shown that this chimera can bind directly to pln HEV. Previous work converting a member of the immunoglobulin superfamily, CD4, to an IgG-like molecule or "immunoadhesin" with potential as an anti–Human Immunodeficiency Virus drug revealed the utility of this procedure in generating new types of "antibody-like" molecules with tailor-made specificities (9). The work described here establishes that nonimmunoglobulin superfamily receptors, such as the mHR, can also be converted to mAb-like molecules. The novelty of this approach is severalfold. First, it allows for the immunohistochemical analysis of the distribution of a ligand for a cell adhesion receptor, even in the absence of an mAb specific for the ligand. In particular, the reagent described in this study, may be useful in mapping the vascular sites in the body where lymphocytes and perhaps other leukocytes bearing the receptor can traffic. Secondly, it is possible that this chimeric molecule may find utility as an antinflammatory reagent by virtue of its ability to block the binding of leukocytes to endothelium (see below). Third, the IgG chimera may be exploited as an affinity reagent to isolate endothelial ligands. Finally, the fact that a nonimmunoglobulin superfamily member was successfully produced and employed here suggests that this procedure may be of general applicability.

Our histochemical analysis with the mHR-IgG chimera definitively establishes that this adhesion molecule can bind directly to the endothelial cells of pln HEV, thus extending the previous finding that spleen-derived mHR can block the binding of lymphocytes to pln HEV (17). The observed staining was over the entire thickness of the endothelial cells, but was frequently concentrated over the apical aspect of the cells where initial contact with lymphocytes is made. Conceivably, the uniform staining represents a cytoplasmic precursor form of the ligand, whereas the apical staining signifies a polarized cell surface expression of the ligand. A fine structural analysis, using the chimera for EM immunocytochemical localization, is required to provide a detailed description of the ligand's localization on the cell surface and within the cell. While the nature of the ligand is currently unknown, previous work has identified a pln endothelial antigen that is recognized by the adhesion-blocking mAb MECA-79, and may, therefore, function as a HEV ligand for lymphocytes (48). Whether this antigen is the cognate ligand of the mHR-IgG chimera or is sterically close to the ligand is a subject for future investigation.

The predominant lack of staining of pp HEV by the mHR-IgG chimera and the failure of the chimera to block lymphocyte binding to this endothelium in vitro was anticipated from previous results (15, 17, 20). The findings reported herein provide the first direct confirmation that the endothelial ligand for the pln mHR is distributed in an organ-restricted manner. Presumably, the ligand for the pp HR will have the converse distribution. Interestingly, in some instances, we observed moderate staining of pp HEV with the chimera. This observation may represent the existence of dual HEV-ligands within a single lymphoid organ, a situation that is known to occur in mesenteric lymph nodes (8, 35, 47). Presumably, a distinctive milieu of locally produced soluble factors (e.g., cytokines) is responsible for induction and maintenance of each of the organ-restricted HEV-ligands (12, 19). Perhaps, during certain immune responses or in-

flammatory processes, these signals are altered, and the regional specificities become obscured. Additional work will be required to address the physiologic or pathophysiologic significance of the expression of pln HR ligands on pp HEV when it occurs.

As noted above, the ability to use the mHR-IgG chimera as an immunohistochemical reagent now permits us to investigate the relationship between the expression of ligands on endothelial cells in various regions and the ability of leukocytes to extravasate in these regions. Of particular interest is the possible induction of chimera reactive ligands on endothelial cells at sites of acute or chronic inflammation. Motivating this interest is the evidence implicating the involvement of MEL-14 defined glycoproteins on both neutrophils and monocytes in endothelial adhesion during inflammatory processes (24, 25, 29). If the ligand were detected on endothelial cells at a particular site of inflammation, the chimera should then be evaluated for its ability to inhibit the inflammatory response in vivo. The demonstrated activity of the chimera in blocking the in vitro adherence of lymphocytes to HEV supports the possibility that leukocyte–endothelial interactions preceding inflammatory responses might be blocked in vivo. However, the likely involvement of a multiplicity of parallel adhesion systems in leukocyte–endothelial interactions during inflammatory reactions (1, 3, 13, 18, 24, 25, 30, 40, 50) may limit the efficacy of any particular blocking reagent as an antiinflammatory drug. Nonetheless, the chimeric protein described here, or drugs that mimic its ligands, merit consideration as potential therapeutic reagents against inflammatory diseases.

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