AFFIDAVIT OF SPENCER J. JOHNSON

I, Spencer J. Johnson, declare as follows:

1. I am an associate at the law firm of Rothwell, Figg, Ernst & Manbeck P.C.

2. I was asked to obtain a copy of the Volume 9, No. 5 issue of DNA and Cell Biology which contained the article "Expression and Characterization of Human CD4: Immunoglobulin Fusion Proteins" by Zettlmeissl et al.

3. I placed an order with a professional document retrieval vendor, Reprints Desk, with a request for a scanned physical version of this issue and article, including a scan of the cover of the issue and the copyright page. I have used Reprints Desk as a professional document retrieval vendor on many occasions, and in my experience their regular business activities include obtaining electronic and physical copies of publications.

4. A copy of the Zettlmeissl article provided to me by Reprints Desk is attached as Appendix 1.

5. The Volume 9, No. 5 issue of DNA and Cell Biology, on its cover and on its table of contents page, indicates that it is an issue from June 1990. The specific physical copy of the issue sourced by Reprints Desk also bears a sticker from the Health Sciences Library of the University of Wisconsin that is stamped with the date July 9, 1990.

6. I also directed that a copy of the same volume and issue of the Zettlmeissl article be obtained from another professional document retrieval vendor, Thomson Reuters Court Express. While I had not used this vendor previously, their website holds out article retrieval and library copying as being among their regular business activities.

7. A copy of the Zettlmeissl article provided to me by Thomson Reuters Court Express and an accompanying declaration from an employee of Thomson Reuters is attached as Appendix 2.

8. The specific physical copy of the issue sourced by Thomson Reuters bears a stamp from the Library of Congress Copyright Office that is dated July 10, 1990.

9. I was also asked to source the Volume 110, No. 6 issue of The Journal of Cell Biology containing the article "A homing receptor-IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules" by Watson et al.

10. I obtained a copy of the article from the website of the journal's publisher, The Rockefeller University Press. A copy of the Watson article that I downloaded on July 27, 2017 is attached as Appendix 3.

11. Below is a screenshot that I took on July 27, 2017 of the publisher's website (http://jcb.rupress.org/content/110/6/2221/tab-article-info), which indicates that the Watson article and issue were published on June 1, 1990.

Article

A homing receptor-IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules.

S R Watson, Y Imai, C Fennie, J S Geoffroy, S D Rosen, L A Lasky

DOI: 10.1083/jcb.110.6.2221 | Published June 1, 1990

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12. I also directed that a scanned physical copy of the Watson article be obtained through an inter-library loan process. A copy of the Watson article was obtained via the inter-library loan process from Randolph College. The copy,

along with an accompanying declaration from an employee of Randolph College's Lipscomb Library, is attached as Appendix 4.

13. The specific physical copy of the issue from Randolph College bears a stamp from the Lipscomb Library dated June 14, 1990.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code. I also understand that any willful false statement may jeopardize the results of the proceedings in which this declaration is submitted.

Respectfully Submitted,

August 1, 2017
Date

Spencer J. Johnson

APPENDIX 1

VOLUME 9 NUMBER 5 JUNE 1990

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VOLUME 9 NUMBER 5 JUNE 1990

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Expression and Characterization of Human CD4: Immunoglobulin Fusion Proteins

GERD ZETTLMEISSL, JENS-PETER GREGERSEN, JEAN MICHEL DUPORT, SABINE MEHDI, GÖTZ REINER, and BRIAN SEED*

ABSTRACT

Different chimeric antibody-like molecules consisting of the four human CD4 extracellular domains (amino acids 1-369) fused to different parts of human IgG_1 and IgM heavy-chain constant regions have been created and expressed in mammalian cells. For both IgG_1 and IgM fusion proteins, the best expression in COS cells was observed for molecules lacking the CH1 domain of the heavy-chain constant region. The chimeric molecules are potent inhibitors of human immunodeficiency virus (HIV) infection and HIV-mediated cytotoxicity. A CD4:IgG_1 hinge fusion protein, which was analyzed in more detail, binds efficiently to HIV gp160 and human Fc receptors and shows complement-assisted inhibition of viral propagation in culture. Half-life studies after intravenous application of the latter human fusion protein into mice and monkeys showed significant prolongation of serum survival compared to soluble CD4. An IgG_{2b} murine homolog of the human CD4:IgG_1 hinge fusion protein was prepared and evaluated in mice, where it was found to be nontoxic and to have no detectable effect on the humoral response to soluble antigen.

INTRODUCTION

MOST PATIENTS infected with the human immunodeficiency virus (HIV) develop acquired immunodeficiency syndrome (AIDS) (Curran *et al.*, 1988), which is characterized by the progressive depletion of T cells expressing CD4, the cellular receptor for HIV (Sattenau and Weiss, 1988). Soluble CD4 molecules have been shown to interfere with HIV-1 infection and HIV-mediated syncytium formation *in vitro* (Smith *et al.*, 1987; Deen *et al.*, 1988; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Traunecker *et al.*, 1988; Clapham *et al.*, 1989). Recent trials in monkeys have shown that soluble CD4, if administered to infected animals without marked CD4 cell cytopenia, can reduce simian immunodeficiency virus (SIV) titers and improve *in vitro* measures of myelopoiesis (Watanabe *et al.*, 1989). However the SIV titer was found to rise and myelopoietic potential to decline after treatment was discontinued, suggesting that continuous lifetime treatment might be necessary to stave off progressive encroachment of the immune system.

In vitro, CD4 immunotoxin conjugates or fusion proteins have been shown to actively target infected cells for killing (Chaudhary *et al.*, 1988; Till *et al.*, 1988). Unfortunately, because toxins are foreign proteins, they are likely susceptible to immune recognition and clearance if administered repeatedly in a clinical setting.

The ideal immunotoxin would combine natural immune effector function with a specific recognition element directed against the pathogen of interest. If the recognition element is the receptor by which the pathogen gains entry to its host cells, the pathogen cannot mutate away from the immunotoxin and still retain its virulence. Recently, Capon *et al.* (1989) published the expression and properties of CD4:human IgG_1 fusion proteins bearing the CH1 domain, whereas Traunecker *et al.* (1989) showed the expression and characterization of CD4:mouse IgG_{2a} and CD4:mouse IgM molecules lacking the CH1 domain.

In this report we investigate the expression and secretion in mammalian cells and the quaternary structure of CD4:human IgG_1 and CD4:human IgM chimeras harboring the extracellular domain of CD4 and different amounts of the immunoglobulin heavy-chain constant region. We present evidence that chimeras consisting of the CD4 extracellular

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domain fused to the various human antibody effector regions are potent inhibitors of HIV infectivity. The properties of the most efficiently secreted CD4:IgG_1 hinge fusion protein were analyzed in more detail. This molecule binds to HIV gp160 and to different human Fc receptors and exhibits long serum half-lives in mice and monkeys. In long-term assays it shows complement-assisted restriction of viral propagation. Administration of the molecule and its mouse L3T4:IgG_{2b} homolog to mice does not compromise their ability to respond to soluble antigen.

MATERIALS AND METHODS

Purification of fusion proteins from COS cells

IgG_1 fusion proteins were adsorbed in batches to Protein A Tris-acryl (Pierce), harvested by filtration, washed in a column with at least 20 volumes of PBS containing 1% Nonidet P-40, with 5 or more volumes of 0.15 *M* NaCl, 1 m*M* EDTA pH 8, and were eluted with 0.1 *M* acetic acid. IgM fusion proteins were similarly harvested and washed after adsorption to agarose beads coupled (by CNBr activation) with 1 mg/ml of rabbit anti-human IgG affinity-purified antibody (Calbiochem). The anti-IgM agarose columns were eluted with 0.2 *M* glycine-HCl buffer pH 2.5. Eluted fusion proteins were neutralized with 2.0 *M* Tris base and concentrated in centrifugal ultrafilters (Amicon).

Stable BHK cell line transfectants

BHK cells in DME/10% fetal bovine serum (FBS) were transfected with the CD4:IgG_1 hinge fusion gene (20 μg), pSV2dhfr (5 μg), and pRMH140 (neo^r, 5 μg) as described (Zettlmeissl *et al.*, 1988). Three days later, cells were split 1:3 to 1:4 into DME/10% FBS containing 400 μg/ml G-418 and 1 μM methotrexate. Individual colonies were picked and screened for relative expression, and a single isolate (BHK-UC3) was expanded for further analysis. To purify fusion proteins, conditioned culture supernatants of BHK-UC3 cells grown in protein-free medium were filtered, adjusted to 19 m*M* Tris HCl pH 8.6, and adsorbed to a Protein A–Sepharose column. The column was washed with 10 volumes of 150 m*M* NaCl, 50 m*M* Tris-HCl pH 8.6, and eluted with 0.1 *M* sodium citrate pH 3. The eluate was adjusted to pH 8 with Tris base and dialyzed against 50 m*M* Tris-HCl pH 7.4 containing 50 m*M* NaCl and 1 m*M* EDTA (TNE-buffer). The resulting protein was >95% pure by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Syncytium assay

HPB-ALL cells were infected with the VCS-25 vaccinia recombinant bearing the HIV-1 envelope-coding sequence. Twelve hours post infection, the cells were divided into microtiter wells and purified, or partially purified fusion proteins were added to each well. OKT4a monoclonal antibody was added as a positive control, and mouse L3T4:IgG_{2b} hinge fusion protein or human CD2:IgG_1 hinge fusion protein was added as a negative control. After 8 more hr of incubation, the cells were washed with PBS, fixed with PBS containing 4% paraformaldehyde, and microscopically analyzed for the presence of giant cells. Cultures without giant cells were considered as negative.

gp160 binding assay

Purified recombinant gp160 from vaccinia virus-infected mammalian cells (kindly provided by Transgene and Pasteur Vaccin) was radioiodinated with lactoperoxidase to a specific activity of 4.5 μCi/μg. Sixty nCi of labeled protein were diluted with increasing amounts of unlabeled gp160 and incubated with CD4:IgG_1 hinge fusion protein (2.7 n*M*) in TNE-buffer in a final volume of 0.2 ml. After 1 hr of incubation at 4°C Protein A–Sepharose (50 μl of a 10% suspension in phosphate-buffered saline containing 1% Triton X-100 and 1 m*M* EDTA; PBSTE) was added and allowed to incubate 2 hr further; bound and free fractions were separated by centrifugation of the beads and washing with 200 μl of PBSTE and PBSTE containing 0.2% $NaDoDSO_4$. The data were corrected for nonspecific binding, determined as counts bound in the absence of fusion protein.

RESULTS

CD4:immunoglobulin fusion genes were created by fusing a CD4 cDNA to human IgG_1 and IgM genomic sequences. The fusions were accomplished with the aid of a joining oligonucleotide encoding a 5-amino-acid linker sequence (HADPE) and a synthetic splice donor sequence, which allowed the reading frame to be preserved between CD4 and the various Ig exons to which it was fused (Fig. 1). Five human fusion genes were created in which the portion encoding the extracellular domain of CD4 (amino acids 1–369) and the 5-amino-acid linker sequence were placed upstream from the CH1, hinge, or CH2 exons of the human IgG_1 gene, or upstream from the CH1 or CH2 exons of the IgM gene. Four similar constructs were prepared from the mouse L3T4 gene and murine IgG_{2b} or IgM chromosomal sequences; however, an L3T4 fusion to CH2 was not created. Transient expression of the fusion proteins in COS cells resulted in the secretion of monomeric (CH2 fusion) or dimeric (CH1 and hinge fusion) immunoglobulin-like molecules in the human IgG_1 case, and large multimeric molecules beyond the resolution of the gel system used in the human IgM case (Fig. 2A). In general, poor expression was observed for fusion proteins bearing CH1 domains from either murine or human immunoglobulins (Fig. 2 and data not shown).

To prepare large amounts of a highly expressed fusion protein, a BHK cell stable transfectant was prepared from the CD4:IgG_1 hinge fusion construct. From the transfectant supernatant a final purified yield of 5–15 μg/ml/day was obtained. Gel electrophoretic analysis of the purified protein showed dimeric molecules, with an apparent subunit molecular mass of 75 kD (Fig. 2B). Equilibrium analytical ultracentrifugation showed a single molecular species

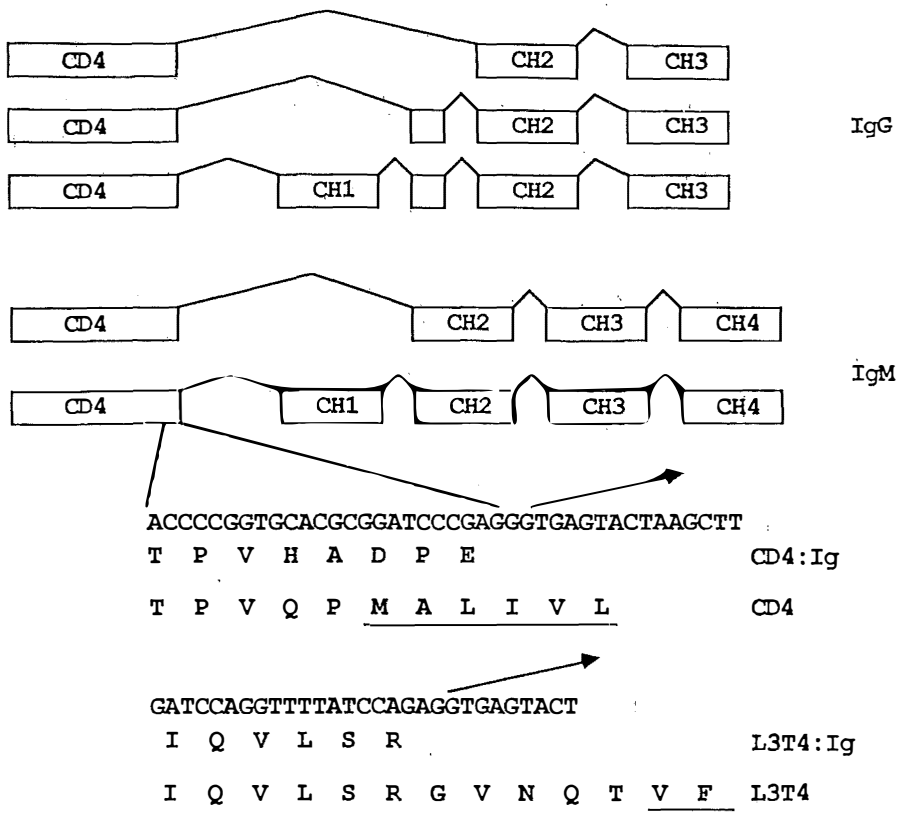

FIG. 1. Structure of the human and murine Ig fusion genes. Human (CD4) and murine (L3T4) synthetic splice donor sequences and their translation products are shown below a schematic diagram of the fusion genes. The native transmembrane domain is underlined.

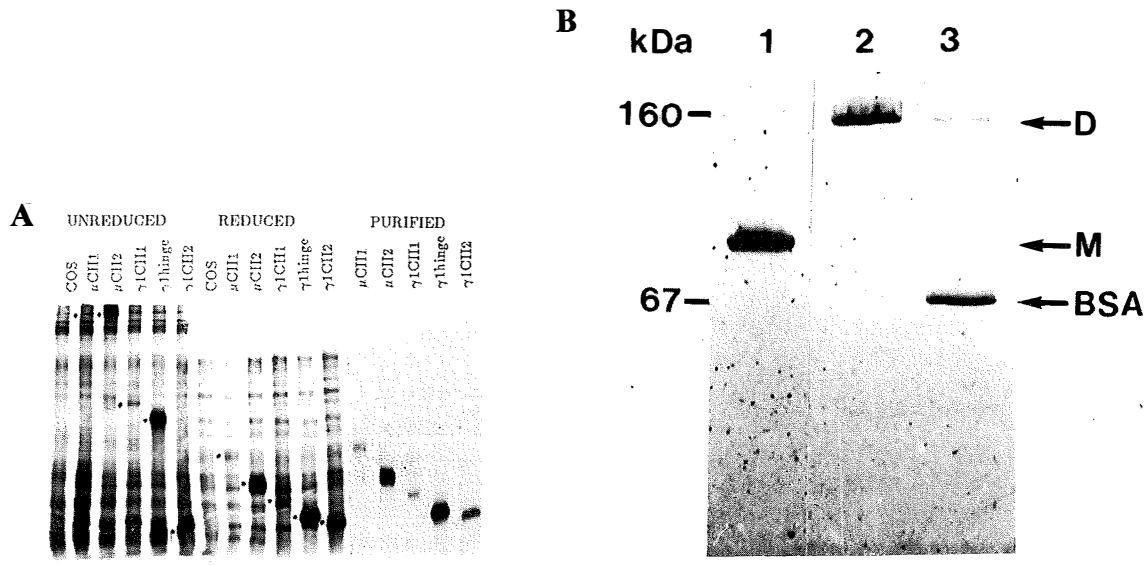

FIG. 2. Expression of the human fusion genes in COS cells (a) and BHK cells (b). a. Cells were transfected with the indicated CD4:Ig fusion genes cloned in a CDM8-based expression vector (Seed, 1987) as previously described (Seed and Aruffo, 1987). At 40 hr post transfection, the medium was aspirated and replaced by serum-free DME medium lacking cysteine and methionine (GIBCO), containing 200 μCi/ml of [^{35}S]methionine and $\approx$40 μCi/ml of [^{35}S]cysteine (Translabel, ICN). Eight hours later, the medium was removed and aliquots, either reduced or nonreduced, were applied to a 5% polyacrylamide gel. Fusion proteins were purified as described in the Materials and Methods. b. CD4:IgG$_1$ hinge fusion protein prepared from a stable BHK cell transfectant. Reduced (lane 1) and unreduced (lane 2) fusion protein prepared from BHK cells are displayed alongside to mouse IgG and BSA markers (lane 3).

with a mass of 145 ± 12 kD (R. Jaenicke, personal communication). Amino-terminal sequence analysis yielded KKVVLGKKGD (B. Siebold, personal communication) in accord with the known sequence.

Pure or partially purified proteins secreted from COS cells were assayed for inhibition of syncytia formed between HPB-ALL (T-cell leukemia) cells infected with a vaccinia virus recombinant (Chakrabarti *et al.*, 1986) bearing the HIV envelope coding region. Blockade of syncytium formation was observed at a concentration of 20 μg/ml for all but the IgG_1 CH1 fusion protein, whose limited availability allowed testing only at 5 μg/ml. At this concentration, partial blockade was observed. The CD4:IgG_1 hinge fusion protein purified from BHK cells was analyzed in more detail and also proved effective at blocking HIV-1 replication in a long-term virus neutralization assay over 5 weeks in Jurkat cells. Cultures with either reverse transcriptase activity (Gregersen *et al.*, 1988) or giant cell formation were considered as positive. The 50% neutralizing concentration for infection of cultures with 100 tissue culture infectious doses (TCID) was 1 μg/ml, and the inclusion of guinea pig complement provided complete neutralization at this concentration (data not shown). Moreover, the BHK-prepared CD4:IgG_1 hinge fusion protein efficiently suppressed the spread of virus from previously infected cells to uninfected cells cocultured with them for long periods of time (Table 1). When infected cultures were treated with CD4 fusion protein in the presence of complement for extended periods of time, a decrease in reverse transcriptase activity was seen over that measured in cultures treated with CD4 fusion protein alone (Table 1).

Despite this, neither the IgG nor the IgM fusion proteins displayed significant complement-dependent activity in a short-term chromium release assay with cells infected by a vaccinia recombinant expressing HIV envelope sequences (data not shown).

The ability of the purified CD4:IgG_1 hinge protein to bind to human macrophage high- and low-affinity Fc receptors was measured by displacement analysis of the binding of radiolabeled IgG_1 to COS cells transfected with cDNAs encoding FcRI, FcRIIa, or FcRIIb (Stengelin *et al.*, 1988; Allen and Seed, 1989). The CD4 fusion protein showed an affinity for the three receptors that was the same, or slightly higher, than the affinity for IgG_1 itself (Fig. 3A–C). In spite of this result, we observed no enhancement of infection of human peripheral blood macrophages (monitored by an antigen ELISA for HIV-1/p24) when infectious doses of HIV-1 (5–80 $TCID_{50}$) were preincubated with up to 50 μg/ml of the CD4:IgG_1 hinge protein (for experimental details, see Gregersen *et al.*, 1990).

Scatchard analysis of the binding of ^{125}I-labeled recombinant gp160 gave a dissociation constant of 7.6 ± 1.7 × 10^{-9} *M* (Fig. 3D), which relates very closely with published dissociation constants for soluble recombinant CD4 (Capon *et al.*, 1989).

Because antibodies are among the longest lived of circulation proteins, CD4:Ig fusion proteins might be expected to show increased serum survival. To test this, purified protein prepared from the BHK transfectant was injected intravenously into mice and cynomolgus monkeys (*Macacca fascicularis*). Persistence of the intact fusion protein was measured by a specific capture ELISA assay using solid-phase anti-CD4 antibody and an anti-immunoglobulin solution phase second antibody reagent. The plasma half-life of the protein determined from the asymptotic decay behavior was 12 hr in monkeys and 14 hr in mice (Fig. 4). These values are about 50-fold higher than the re-

TABLE 1. EFFECT OF CD4:IgG_1 HINGE FUSION PROTEIN ON HIV-1-INFECTED CELLS

Treatment on days 0, 8, and 11 with:	*CD4:IgG_1 (10 μg/ml)*		*CD4:IgG_1 (10 μg/ml) + complement*		*Complement*		*TNE buffer*	
Infection parameters:	*RT*[a]	*Fusions*	*RT*	*Fusions*	*RT*	*Fusions*	*RT*	*Fusions*
Day 0	ND[b]	+	ND	+	ND	+	ND	+
Day 3	2.8	–	1.5	–	2.1	+ +	3.8	+ + +
Day 7	2.0	+	1.8	+	4.3	+ +	12.9	+ + +
Day 10	2.7	+	1.5	+	16.4	+ +	49.9	+ + +
Day 14	2.2	+	1.0	+	26.8	+ +	31.3	+ + +
Day 17	1.8	+	1.0	–	25.6	+ +	20.7	+ +
Day 21	2.3	+	1.8	–	27.0	+ +	21.3	+ +
Day 24	2.5	+	0.7	–	13.3	+ +	10.5	+ +
Day 28	7.7	+	1.1	–	9.6	+	7.4	+

[a]RT, Reverse transcriptase activity.
[b]ND, Not determined.

Infected H9 cells were mixed 1:10 into cultures of uninfected H9 cells (10^7 cells in 10 ml). Duplicate cultures were treated with CD4:IgG_1 hinge fusion protein, guinea pig complement (1:30), a combination of both, and TNE-buffer as control at the indicated days. Fifty percent of the culture medium of all cultures was replaced twice weekly, and once a week up to 50% of the cells were removed to adjust cell densities to constant values between 1–2 × 10^6 cells/ml.

Reverse transcriptase activity was determined according to Gregersen *et al.* (1988) and is given as sample/control ratio using supernatants of uninfected cultures as control. Sample/control ratios above 2 are considered as positive.

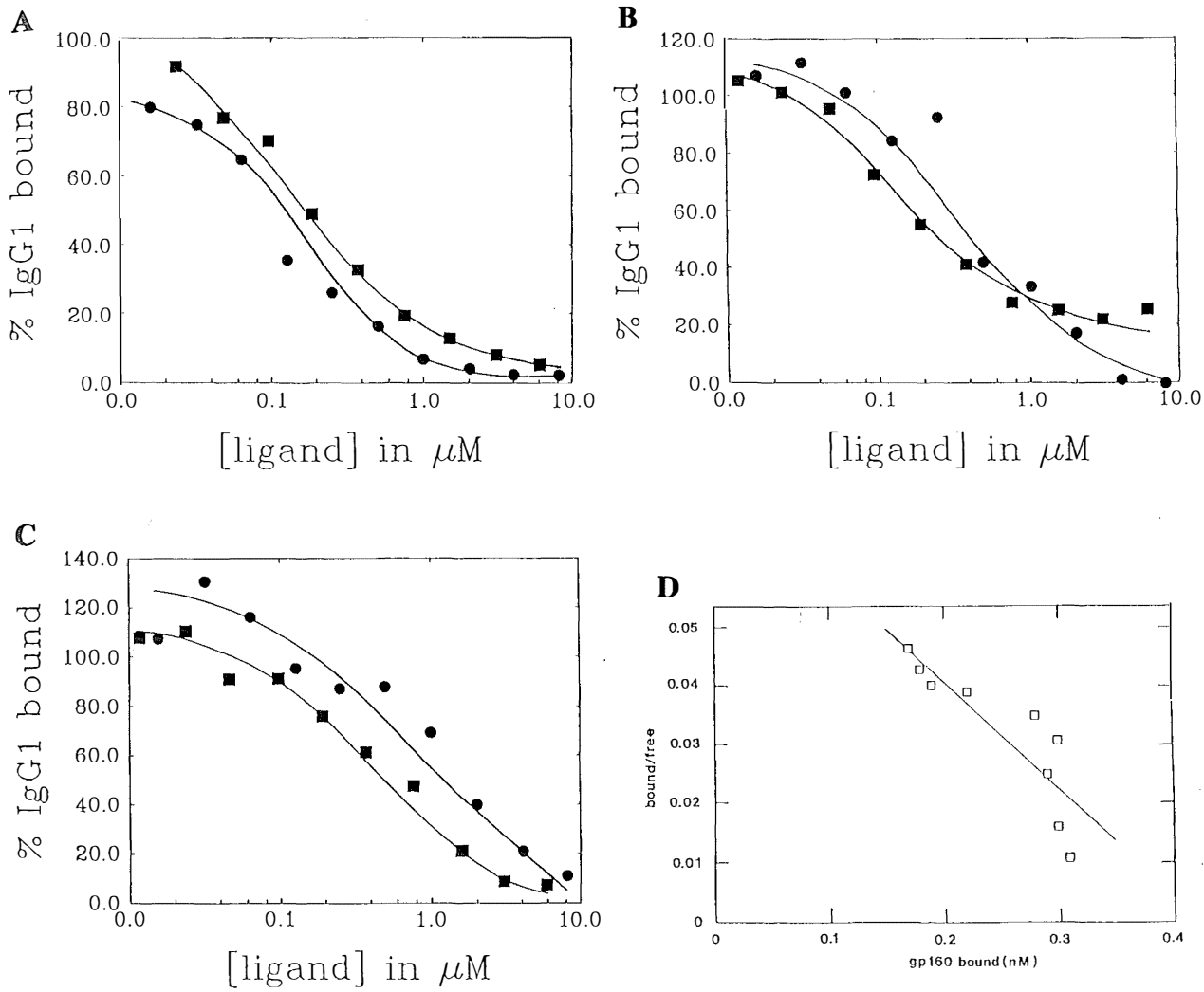

FIG. 3. CD4:IgG_1 hinge fusion protein binding analyses. a–c. Displacement analysis of binding of CD4:IgG_1 hinge fusion protein to Fc receptors expressed by COS cells. Displacement of ^{125}I-labeled human IgG_1 from COS cells transfected with high- (FcRI) (a) or low- (FcRIIa, FcRIIb) (b,c) affinity receptors was carried out as described (Stengelin *et al.*, 1988). (●) Displacement by fusion protein; (■) displacement by unlabeled IgG_1. d. Scatchard analysis of CD4 fusion protein binding to labeled HIV gp160. The solid line represents the best fit by least-square regression analysis to the mean values of five independent determinations.

ported value for the half-life of soluble CD4 in rabbits, but about 4-fold lower than reported for a fusion protein bearing the first two domains of CD4 fused to the human IgG_1 CH1 domain (Capon *et al.*, 1989).

Preliminary toxicological studies were carried out with human and murine fusion proteins in mice. On day 1, groups of 10 mice (5 male and 5 female) were injected intravenously with 80 μg per animal of either purified human CD4:IgG_1 hinge fusion protein, purified murine L3T4:IgG_{2b} hinge fusion protein, or phosphate-buffered saline. The animals were observed for 30 days and were weighed on study days 0, 1, 7, and 14. No adverse reactions or change in body weight were observed. On day 2, 7, and 17, blood cell numbers including leukocytes, erythrocytes, platelets, hemoglobin, hematocrit, and mean cell volume were determined in 2 animals of each group; here too, no significant changes were observed. On day 14 the animals were reinjected as on day 1. On day 16 the animals were immunized with tetanus toxoid (TETANOL, Behringwerke AG, Marburg, FRG; 0.5 ml by intraperitoneal injection). Anti-toxoid antibody titers of sera collected on day 30 were determined by a specific ELISA. Control animals showed end point dilution values of 1:11,900 ± 5,500, while animals injected with the human and murine fusion proteins gave end point dilution values of 1:14,100 ± 6,400 and 1:9,200 ± 4,600, respectively.

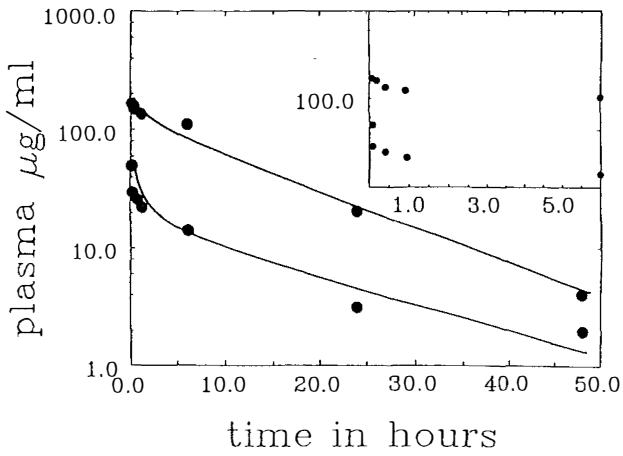

FIG. 4. Serum half-life of human CD4:IgG_1 hinge fusion protein in mice (lower) and monkeys (upper). The inset shows the first five samples of each experiment in expanded scale. For the mouse experiment, two groups of 3 animals were injected in the tail vein with 100 μg of purified CD4:IgG_1 hinge fusion protein per mouse. From one group, blood samples were collected by retro-orbital bleeds 5 min, 30 min, 6 hr, and 48 hr after injection; from the other, samples were collected 10 min, 1 hr, and 24 hr after injection. For the monkey experiment, two cynomolgus monkeys with a body weight of 1.4 kg were injected in the saphenous vein with a bolus of 5 mg of purified fusion protein. One-milliliter blood samples were collected from the femoral vein at the same time intervals as for the mouse experiments. Serum concentrations of fusion protein were measured by a specific capture ELISA. Serum samples were incubated in microtiter wells coated with anti-CD4 antibody BMA041 (Behringwerke). The wells were washed, and developed with an alkaline phosphatase coupled antibody to human IgG (Zymed). The data for all animals are pooled in the figure shown.

DISCUSSION

Chimeric proteins bearing the extracellular domains of cell-surface proteins fused to immunoglobulin constant domains show promise as therapeutic agents. One of the most important issues confronting these agents is the extent of autoimmune damage arising from interaction of the fusion protein with its native ligand. Fortunately, the affinity of CD4 for its presumptive natural ligand, HLA class II antigen, is very low, and the affinity for HIV envelope is quite high. We find here, using a mouse model, that the fusion molecules have low toxicity in animals, and do not interfere with the humoral response to soluble antigen *in vivo*, consistent with the expectations raised by *in vitro* studies.

High-level expression of immunoglobulin fusion proteins was obtained following either transient or stable expression in nonlymphoid cells. These observations confirm and extend previous reports of immunoglobulin expression in transfected cells (Cattaneo and Neuberger, 1987; De Waele *et al.*, 1988), and show that high-molecular-weight IgM-like complexes can be formed in the absence of the J chain. Previous studies of IgM expression by nonlymphoid transfectants have shown that absence of the J chain allows hexameric IgMs to accumulate, rather than the nearly exclusively pentameric chains found *in vivo* (Cattaneo and Neuberger, 1987). The presence of either IgG or IgM CH1 domains correlated with markedly reduced fusion expression, a result not entirely unexpected, given that CH1 is normally found associated with either a light-chain constant region or heavy-chain binding protein during the course of synthesis in B cells (Haas and Meo, 1988).

The purified fusion proteins potently inhibited virus infection and dissemination *in vitro*, and cultures treated with both fusion protein and complement fared better than cultures treated with either fusion protein alone or complement alone. In contrast, neither the IgG_1 nor the IgM fusion proteins showed significant complement-mediated cytotoxicity in a short-term chromium release assay. These results suggest that short-term assays may not measure relatively subtle or slow-acting effects that play an important role in long-term cultures, or that complement may participate in virus restriction by a mechanism divorced from cytolysis, *e.g.*, through interaction with specific receptors.

The inability to demonstrate a rapid direct cytolysis, despite high-affinity interaction and apparently normal binding of initial complement components (M. Leineweber and B. Seed, unpublished), is not unexpected. Even neutralizing antisera to HIV envelope determinants rarely show direct cytolytic activity in short-term *in vitro* assays (Nara *et al.*, 1987). Further, unlike most murine retroviruses, human retroviruses HIV-1 and HTLV-1 are little affected by exposure to human serum (Hoshino *et al.*, 1984; Banapour *et al.*, 1986). In a similar study (Capon *et al.*, 1989), it has been reported that a CD4:human IgG_1 CH1 fusion protein did not bind complement component C1q, and bound only to the high-affinity Fc receptors of the U937 cell line, which bears approximately twice as many low-affinity as high-affinity receptors (Vaughn *et al.*, 1985; Looney *et al.*, 1986; Fanger *et al.*, 1989). The reasons for these discrepancies are not known, but may be related to the presence of the CH1 domain, Traunecker *et al.* (1989), for example, found that a CD4:mouse IgG_{2a} hinge fusion protein binds C1q. Further detailed analysis of the fusion proteins in HIV/SIV *in vitro* and *in vivo* models (see, for example, Watanabe *et al.*, 1989) will provide data about whether these molecules represent a realistic approach to AIDS therapy.

ACKNOWLEDGMENTS

We thank Sandro Aruffo for participation in the early phases of this project, and for useful suggestions; Rainer Jaenicke for communicating his results on analytical ultracentrifugation; Ernst Weinmann for assistance in the pharmacokinetic measurements; Gabriele Bergmann, Ludwina Preiss, Elke Veit, Ramona Volk, and Heike Wege for technical assistance; and Hans Küpper and Joachim Hilfenhaus for helpful discussions.

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APPENDIX 2

Declaration of Carmen Debord

I, Carmen Debord, declare the following based on my personal knowledge:

1. I have been employed by Thomson Reuters for 3 years, and I currently hold the title of Senior Research Associate. My duties include document retrieval and legal research at the courts and governmental agencies in Washington, D.C. As such, I am familiar with the process of searching for and locating documents in a library.

2. I was asked to locate a physical copy of the June 1990 issue of DNA and Cell Biology (Vol. 9, No. 5) which contained the article "Expression and Characterization of Human CD4: Immunoglobulin Fusion Proteins" by Zettlemeissl et al.

3. When searching for copies of journals such as DNA and Cell Biology, I would expect to locate them in a library. As is typical for a request for this sort of journal, I was able to find a copy of the requested issue of DNA and Cell Biology indexed in the online catalog of the Library of Congress, which indicated that a copy of the desired volume was available to request. I placed a request for access to the copy. I then visited the Library of Congress, where a librarian provided me with the copy.

4. The issue of DNA and Cell Biology I received was bound into a larger volume. The issue itself was in the condition I expected to find it. On viewing the issue, I found no reason to suspect that it had been altered or was otherwise inauthentic. The table of contents page of the issue bears a stamp from the Copyright Office dated July 10, 1990, which indicates to me that the issue was received or processed by the Library of Congress on July 10, 1990.

5. After determining that I had located the issue of DNA and Cell Biology that had been requested, I scanned the cover of the issue, the initial pages of the journal up through the table of contents, and the article by Zettlemeissl which had been requested.

6. A copy of the scanned journal issue and article is attached as Appendix A to this declaration.

7. The scanned journal issue and article are as they were provided to me by a librarian of the Library of Congress unaltered, including the Library's date of receipt stamp on the table of contents page, which bears the date July 10, 1990.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code. I also understand that any willful false statement may jeopardize the results of the proceedings in which this declaration is submitted.

Respectfully Submitted,

1/22/17
Date

Carmen Debord

APPENDIX A

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Expression and Characterization of Human CD4: Immunoglobulin Fusion Proteins

GERD ZETTLMEISSL, JENS-PETER GREGERSEN, JEAN MICHEL DUPORT, SABINE MEHDI, GÖTZ REINER, and BRIAN SEED*

ABSTRACT

Different chimeric antibody-like molecules consisting of the four human CD4 extracellular domains (amino acids 1–369) fused to different parts of human IgG_1 and IgM heavy-chain constant regions have been created and expressed in mammalian cells. For both IgG_1 and IgM fusion proteins, the best expression in COS cells was observed for molecules lacking the CH1 domain of the heavy-chain constant region. The chimeric molecules are potent inhibitors of human immunodeficiency virus (HIV) infection and HIV-mediated cytotoxicity. A CD4:IgG_1 hinge fusion protein, which was analyzed in more detail, binds efficiently to HIV gp160 and human Fc receptors and shows complement-assisted inhibition of viral propagation in culture. Half-life studies after intravenous application of the latter human fusion protein into mice and monkeys showed significant prolongation of serum survival compared to soluble CD4. An IgG_{2b} murine homolog of the human CD4:IgG_1 hinge fusion protein was prepared and evaluated in mice, where it was found to be nontoxic and to have no detectable effect on the humoral response to soluble antigen.

INTRODUCTION

Most patients infected with the human immunodeficiency virus (HIV) develop acquired immunodeficiency syndrome (AIDS) (Curran *et al.*, 1988), which is characterized by the progressive depletion of T cells expressing CD4, the cellular receptor for HIV (Sattenau and Weiss, 1988). Soluble CD4 molecules have been shown to interfere with HIV-1 infection and HIV-mediated syncytium formation *in vitro* (Smith *et al.*, 1987; Deen *et al.*, 1988; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Traunecker *et al.*, 1988; Clapham *et al.*, 1989). Recent trials in monkeys have shown that soluble CD4, if administered to infected animals without marked CD4 cell cytopenia, can reduce simian immunodeficiency virus (SIV) titers and improve *in vitro* measures of myelopoiesis (Watanabe *et al.*, 1989). However the SIV titer was found to rise and myelopoietic potential to decline after treatment was discontinued, suggesting that continuous lifetime treatment might be necessary to stave off progressive encroachment of the immune system.

In vitro, CD4 immunotoxin conjugates or fusion proteins have been shown to actively target infected cells for killing (Chaudhary *et al.*, 1988; Till *et al.*, 1988). Unfortunately, because toxins are foreign proteins, they are likely susceptible to immune recognition and clearance if administered repeatedly in a clinical setting.

The ideal immunotoxin would combine natural immune effector function with a specific recognition element directed against the pathogen of interest. If the recognition element is the receptor by which the pathogen gains entry to its host cells, the pathogen cannot mutate away from the immunotoxin and still retain its virulence. Recently, Capon *et al.* (1989) published the expression and properties of CD4:human IgG_1 fusion proteins bearing the CH1 domain, whereas Traunecker *et al.* (1989) showed the expression and characterization of CD4:mouse IgG_{2a} and CD4:mouse IgM molecules lacking the CH1 domain.

In this report we investigate the expression and secretion in mammalian cells and the quaternary structure of CD4:human IgG_1 and CD4:human IgM chimeras harboring the extracellular domain of CD4 and different amounts of the immunoglobulin heavy-chain constant region. We present evidence that chimeras consisting of the CD4 extracellular

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domain fused to the various human antibody effector regions are potent inhibitors of HIV infectivity. The properties of the most efficiently secreted CD4:IgG_1 hinge fusion protein were analyzed in more detail. This molecule binds to HIV gp160 and to different human Fc receptors and exhibits long serum half-lives in mice and monkeys. In long-term assays it shows complement-assisted restriction of viral propagation. Administration of the molecule and its mouse L3T4:IgG_{2b} homolog to mice does not compromise their ability to respond to soluble antigen.

MATERIALS AND METHODS

Purification of fusion proteins from COS cells

IgG_1 fusion proteins were adsorbed in batches to Protein A Tris-acryl (Pierce), harvested by filtration, washed in a column with at least 20 volumes of PBS containing 1% Nonidet P-40, with 5 or more volumes of 0.15 *M* NaCl, 1 m*M* EDTA pH 8, and were eluted with 0.1 *M* acetic acid. IgM fusion proteins were similarly harvested and washed after adsorption to agarose beads coupled (by CNBr activation) with 1 mg/ml of rabbit anti-human IgG affinity-purified antibody (Calbiochem). The anti-IgM agarose columns were eluted with 0.2 *M* glycine-HCl buffer pH 2.5. Eluted fusion proteins were neutralized with 2.0 *M* Tris base and concentrated in centrifugal ultrafilters (Amicon).

Stable BHK cell line transfectants

BHK cells in DME/10% fetal bovine serum (FBS) were transfected with the CD4:IgG_1 hinge fusion gene (20 μg), pSV2dhfr (5 μg), and pRMH140 (neo^r, 5 μg) as described (Zettlmeissl *et al.*, 1988). Three days later, cells were split 1:3 to 1:4 into DME/10% FBS containing 400 μg/ml G-418 and 1 μM methotrexate. Individual colonies were picked and screened for relative expression, and a single isolate (BHK-UC3) was expanded for further analysis. To purify fusion proteins, conditioned culture supernatants of BHK-UC3 cells grown in protein-free medium were filtered, adjusted to 19 m*M* Tris HCl pH 8.6, and adsorbed to a Protein A-Sepharose column. The column was washed with 10 volumes of 150 m*M* NaCl, 50 m*M* Tris-HCl pH 8.6, and eluted with 0.1 *M* sodium citrate pH 3. The eluate was adjusted to pH 8 with Tris base and dialyzed against 50 m*M* Tris-HCl pH 7.4 containing 50 m*M* NaCl and 1 m*M* EDTA (TNE-buffer). The resulting protein was >95% pure by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Syncytium assay

HPB-ALL cells were infected with the VCS-25 vaccinia recombinant bearing the HIV-1 envelope-coding sequence. Twelve hours post infection, the cells were divided into microtiter wells and purified, or partially purified fusion proteins were added to each well. OKT4a monoclonal antibody was added as a positive control, and mouse L3T4:IgG_{2b} hinge fusion protein or human CD2:IgG_1 hinge fusion protein was added as a negative control. After 8 more hr of incubation, the cells were washed with PBS, fixed with PBS containing 4% paraformaldehyde, and microscopically analyzed for the presence of giant cells. Cultures without giant cells were considered as negative.

gp160 binding assay

Purified recombinant gp160 from vaccinia virus-infected mammalian cells (kindly provided by Transgene and Pasteur Vaccin) was radioiodinated with lactoperoxidase to a specific activity of 4.5 μCi/μg. Sixty nCi of labeled protein were diluted with increasing amounts of unlabeled gp160 and incubated with CD4:IgG_1 hinge fusion protein (2.7 n*M*) in TNE-buffer in a final volume of 0.2 ml. After 1 hr of incubation at 4°C Protein A-Sepharose (50 μl of a 10% suspension in phosphate-buffered saline containing 1% Triton X-100 and 1 m*M* EDTA; PBSTE) was added and allowed to incubate 2 hr further; bound and free fractions were separated by centrifugation of the beads and washing with 200 μl of PBSTE and PBSTE containing 0.2% $NaDoDSO_4$. The data were corrected for nonspecific binding, determined as counts bound in the absence of fusion protein.

RESULTS

CD4:immunoglobulin fusion genes were created by fusing a CD4 cDNA to human IgG_1 and IgM genomic sequences. The fusions were accomplished with the aid of a joining oligonucleotide encoding a 5-amino-acid linker sequence (HADPE) and a synthetic splice donor sequence, which allowed the reading frame to be preserved between CD4 and the various Ig exons to which it was fused (Fig. 1). Five human fusion genes were created in which the portion encoding the extracellular domain of CD4 (amino acids 1-369) and the 5-amino-acid linker sequence were placed upstream from the CH1, hinge, or CH2 exons of the human IgG_1 gene, or upstream from the CH1 or CH2 exons of the IgM gene. Four similar constructs were prepared from the mouse L3T4 gene and murine IgG_{2b} or IgM chromosomal sequences; however, an L3T4 fusion to CH2 was not created. Transient expression of the fusion proteins in COS cells resulted in the secretion of monomeric (CH2 fusion) or dimeric (CH1 and hinge fusion) immunoglobulin-like molecules in the human IgG_1 case, and large multimeric molecules beyond the resolution of the gel system used in the human IgM case (Fig. 2A). In general, poor expression was observed for fusion proteins bearing CH1 domains from either murine or human immunoglobulins (Fig. 2 and data not shown).

To prepare large amounts of a highly expressed fusion protein, a BHK cell stable transfectant was prepared from the CD4:IgG_1 hinge fusion construct. From the transfectant supernatant a final purified yield of 5-15 μg/ml/day was obtained. Gel electrophoretic analysis of the purified protein showed dimeric molecules, with an apparent subunit molecular mass of 75 kD (Fig. 2B). Equilibrium analytical ultracentrifugation showed a single molecular species

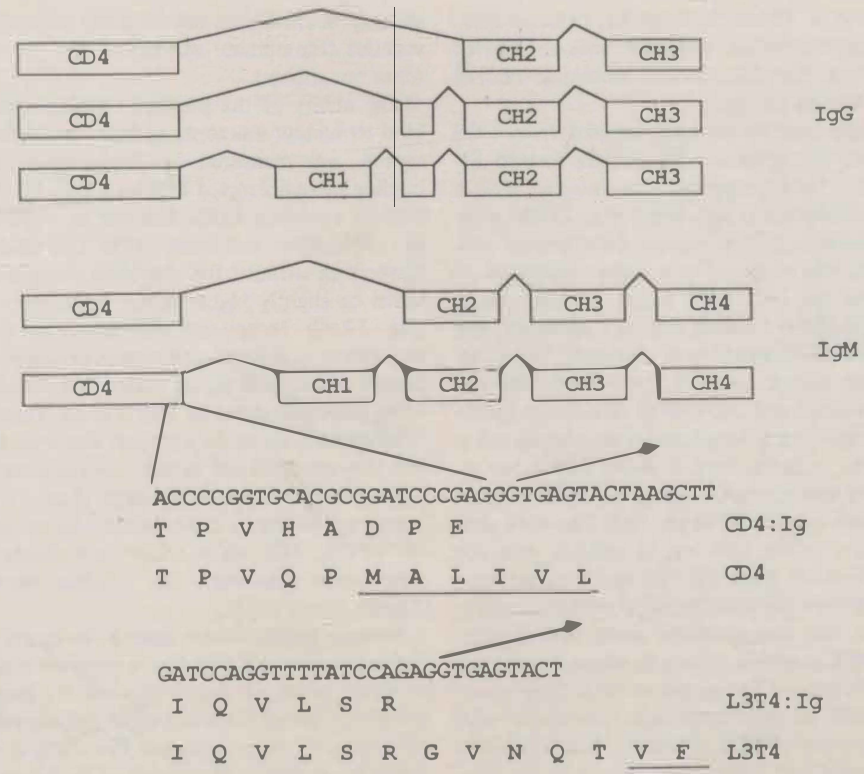

FIG. 1. Structure of the human and murine Ig fusion genes. Human (CD4) and murine (L3T4) synthetic splice donor sequences and their translation products are shown below a schematic diagram of the fusion genes. The native transmembrane domain is underlined.

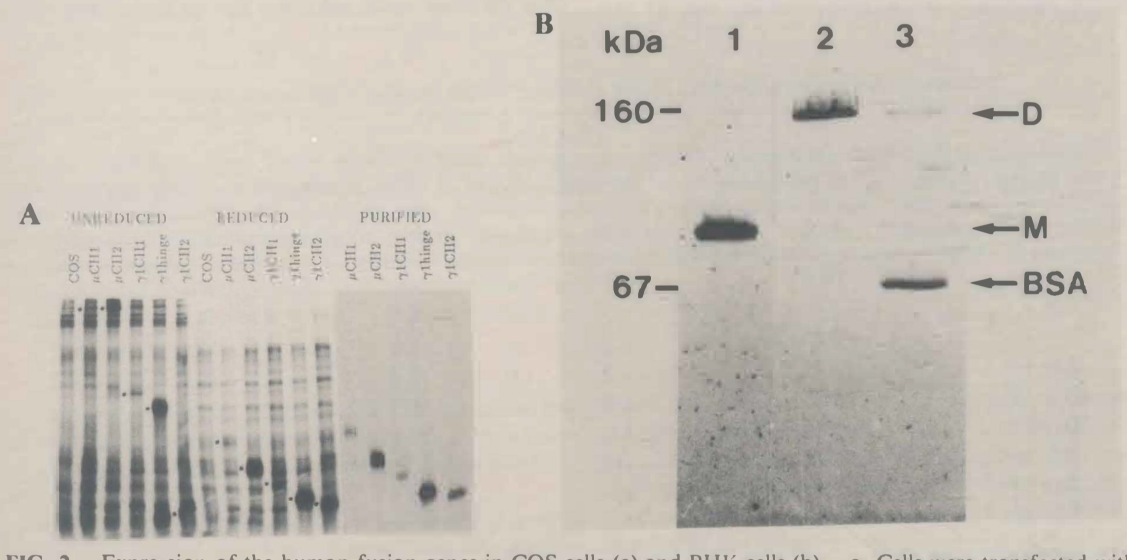

FIG. 2. Expression of the human fusion genes in COS cells (a) and BHK cells (b). a. Cells were transfected with the indicated CD4:Ig fusion genes cloned in a CDM8-based expression vector (Seed, 1987) as previously described (Seed and Aruffo, 1987). At 40 hr post transfection, the medium was aspirated and replaced by serum-free DME medium lacking cysteine and methionine (GIBCO), containing 200 μCi/ml of [^{35}S]methionine and $\approx$40 μCi/ml of [^{35}S]cysteine (Translabel, ICN). Eight hours later, the medium was removed and aliquots, either reduced or nonreduced, were applied to a 5% polyacrylamide gel. Fusion proteins were purified as described in the Materials and Methods. b. CD4:IgG_1 hinge fusion protein prepared from a stable BHK cell transfectant. Reduced (lane 1) and unreduced (lane 2) fusion protein prepared from BHK cells are displayed alongside to mouse IgG and BSA markers (lane 3).

with a mass of 145 ± 12 kD (R. Jaenicke, personal communication). Amino-terminal sequence analysis yielded KKVVLGKKGD (B. Siebold, personal communication) in accord with the known sequence.

Pure or partially purified proteins secreted from COS cells were assayed for inhibition of syncytia formed between HPB-ALL (T-cell leukemia) cells infected with a vaccinia virus recombinant (Chakrabarti *et al.*, 1986) bearing the HIV envelope coding region. Blockade of syncytium formation was observed at a concentration of 20 μg/ml for all but the IgG_1 CH1 fusion protein, whose limited availability allowed testing only at 5 μg/ml. At this concentration, partial blockade was observed. The CD4:IgG_1 hinge fusion protein purified from BHK cells was analyzed in more detail and also proved effective at blocking HIV-1 replication in a long-term virus neutralization assay over 5 weeks in Jurkat cells. Cultures with either reverse transcriptase activity (Gregersen *et al.*, 1988) or giant cell formation were considered as positive. The 50% neutralizing concentration for infection of cultures with 100 tissue culture infectious doses (TCID) was 1 μg/ml, and the inclusion of guinea pig complement provided complete neutralization at this concentration (data not shown). Moreover, the BHK-prepared CD4:IgG_1 hinge fusion protein efficiently suppressed the spread of virus from previously infected cells to uninfected cells cocultured with them for long periods of time (Table 1). When infected cultures were treated with CD4 fusion protein in the presence of complement for extended periods of time, a decrease in reverse transcriptase activity was seen over that measured in cultures treated with CD4 fusion protein alone (Table 1).

Despite this, neither the IgG nor the IgM fusion proteins displayed significant complement-dependent activity in a short-term chromium release assay with cells infected by a vaccinia recombinant expressing HIV envelope sequences (data not shown).

The ability of the purified CD4:IgG_1 hinge protein to bind to human macrophage high- and low-affinity Fc receptors was measured by displacement analysis of the binding of radiolabeled IgG_1 to COS cells transfected with cDNAs encoding FcRI, FcRIIa, or FcRIIb (Stengelin *et al.*, 1988; Allen and Seed, 1989). The CD4 fusion protein showed an affinity for the three receptors that was the same, or slightly higher, than the affinity for IgG_1 itself (Fig. 3A–C). In spite of this result, we observed no enhancement of infection of human peripheral blood macrophages (monitored by an antigen ELISA for HIV-1/p24) when infectious doses of HIV-1 (5–80 $TCID_{50}$) were preincubated with up to 50 μg/ml of the CD4:IgG_1 hinge protein (for experimental details, see Gregersen *et al.*, 1990).

Scatchard analysis of the binding of ^{125}I-labeled recombinant gp160 gave a dissociation constant of 7.6 ± 1.7 × 10^{-9} *M* (Fig. 3D), which relates very closely with published dissociation constants for soluble recombinant CD4 (Capon *et al.*, 1989).

Because antibodies are among the longest lived of circulation proteins, CD4:Ig fusion proteins might be expected to show increased serum survival. To test this, purified protein prepared from the BHK transfectant was injected intravenously into mice and cynomolgus monkeys (*Macacca fascicularis*). Persistence of the intact fusion protein was measured by a specific capture ELISA assay using solid-phase anti-CD4 antibody and an anti-immunoglobulin solution phase second antibody reagent. The plasma half-life of the protein determined from the asymptotic decay behavior was 12 hr in monkeys and 14 hr in mice (Fig. 4). These values are about 50-fold higher than the reported value for the half-life of soluble CD4 in rabbits, but about 4-fold lower than reported for a fusion protein bearing the first two domains of CD4 fused to the human IgG_1 CH1 domain (Capon *et al.*, 1989).

Preliminary toxicological studies were carried out with human and murine fusion proteins in mice. On day 1, groups of 10 mice (5 male and 5 female) were injected intravenously with 80 μg per animal of either purified human CD4:IgG_1 hinge fusion protein, purified murine L3T4:IgG_{2b} hinge fusion protein, or phosphate-buffered saline. The animals were observed for 30 days and were weighed on study days 0, 1, 7, and 14. No adverse reactions or change in body weight were observed. On day 2, 7, and 17, blood cell numbers including leukocytes, erythrocytes, platelets, hemoglobin, hematocrit, and mean cell volume were determined in 2 animals of each group; here too, no significant changes were observed. On day 14 the animals were reinjected as on day 1. On day 16 the animals were immunized with tetanus toxoid (TETANOL, Behringwerke AG, Marburg, FRG; 0.5 ml by intraperitoneal injection). Anti-toxoid antibody titers of sera collected on day 30 were determined by a specific ELISA. Control animals showed end point dilution values of 1:11,900 ± 5,500, while animals injected with the human and murine fusion proteins gave end point dilution values of 1:14,100 ± 6,400 and 1:9,200 ± 4,600, respectively.

TABLE 1. EFFECT OF CD4:IgG_1 HINGE FUSION PROTEIN ON HIV-1-INFECTED CELLS

Treatment on days 0, 8, and 11 with:	*CD4:IgG_1 (10 μg/ml)*		*CD4:IgG_1 (10 μg/ml) + complement*		*Complement*		*TNE buffer*	
Infection parameters:	*RT*[a]	*Fusions*	*RT*	*Fusions*	*RT*	*Fusions*	*RT*	*Fusions*
Day 0	ND[b]	+	ND	+	ND	+	ND	+
Day 3	2.8	–	1.5	–	2.1	+ +	3.8	+ + +
Day 7	2.0	+	1.8	+	4.3	+ +	12.9	+ + +
Day 10	2.7	+	1.5	+	16.4	+ +	49.9	+ + +
Day 14	2.2	+	1.0	+	26.8	+ +	31.3	+ + +
Day 17	1.8	+	1.0	–	25.6	+ +	20.7	+ +
Day 21	2.3	+	1.8	–	27.0	+ +	21.3	+ +
Day 24	2.5	+	0.7	–	13.3	+ +	10.5	+ +
Day 28	7.7	+	1.1	–	9.6	+	7.4	+

[a]RT, Reverse transcriptase activity.
[b]ND, Not determined.
Infected H9 cells were mixed 1:10 into cultures of uninfected H9 cells (10^7 cells in 10 ml). Duplicate cultures were treated with CD4:IgG_1 hinge fusion protein, guinea pig complement (1:30), a combination of both, and TNE-buffer as control at the indicated days. Fifty percent of the culture medium of all cultures was replaced twice weekly, and once a week up to 50% of the cells were removed to adjust cell densities to constant values between 1–2 × 10^6 cells/ml.
Reverse transcriptase activity was determined according to Gregersen *et al.* (1988) and is given as sample/control ratio using supernatants of uninfected cultures as control. Sample/control ratios above 2 are considered as positive.

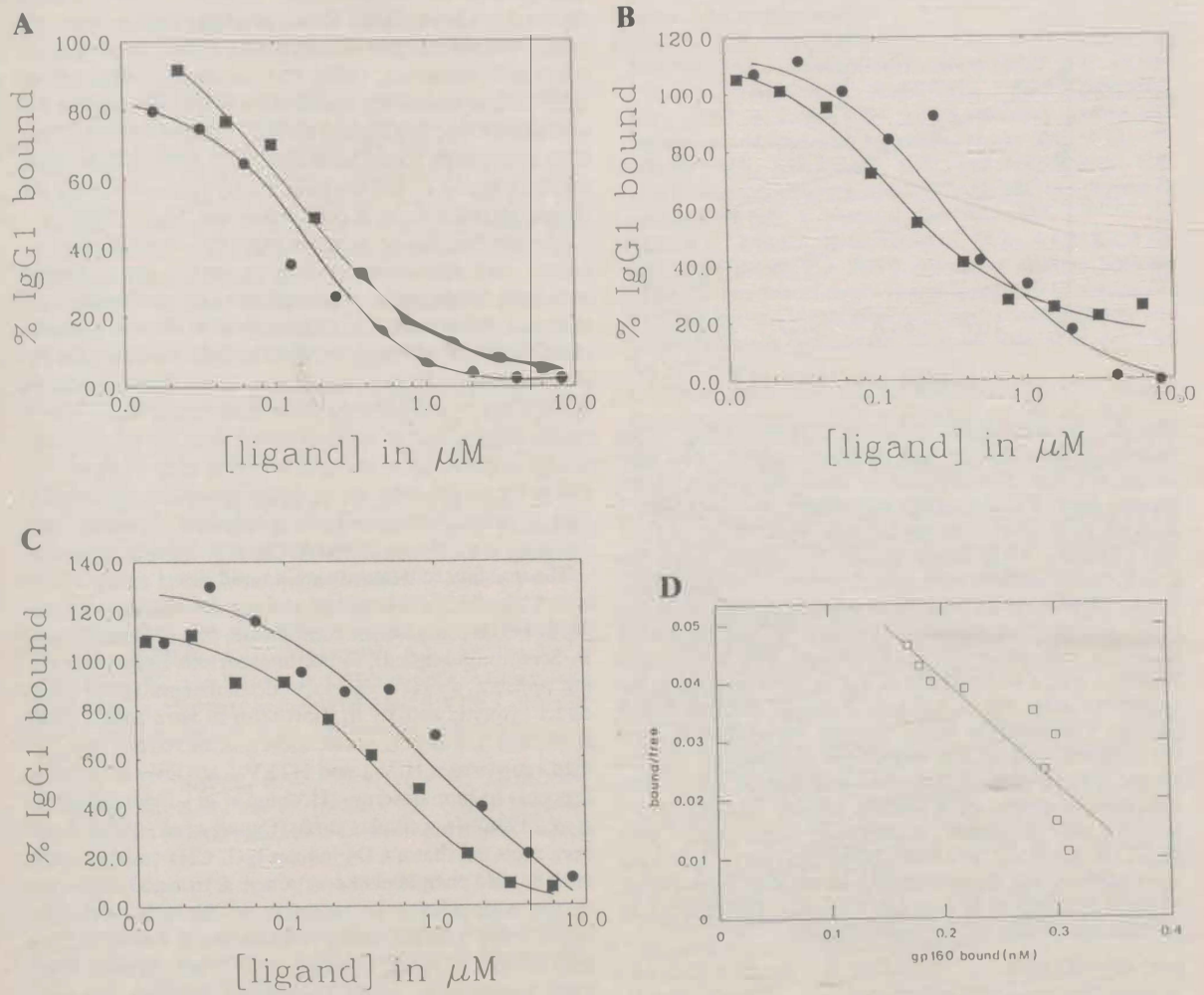

FIG. 3. CD4:IgG_1 hinge fusion protein binding analyses. a–c. Displacement analysis of binding of CD4:IgG_1 hinge fusion protein to Fc receptors expressed by COS cells. Displacement of ^{125}I-labeled human IgG_1 from COS cells transfected with high- (FcRI) (a) or low- (FcRIIa, FcRIIb) (b,c) affinity receptors was carried out as described (Stengelin *et al.*, 1988). (●) Displacement by fusion protein; (■) displacement by unlabeled IgG_1. d. Scatchard analysis of CD4 fusion protein binding to labeled HIV gp160. The solid line represents the best fit by least-square regression analysis to the mean values of five independent determinations.

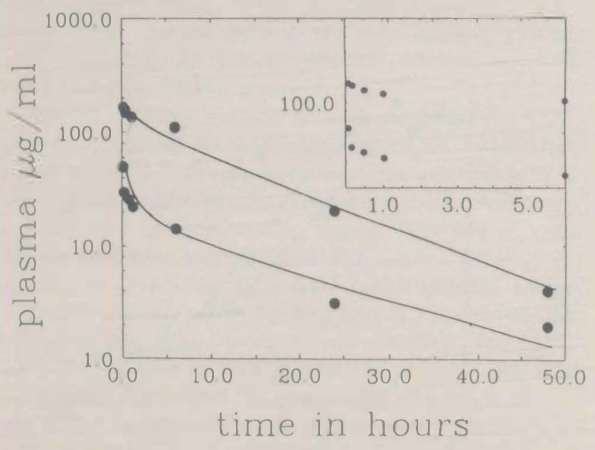

FIG. 4. Serum half-life of human CD4:IgG_1 hinge fusion protein in mice (lower) and monkeys (upper). The inset shows the first five samples of each experiment in expanded scale. For the mouse experiment, two groups of 3 animals were injected in the tail vein with 100 μg of purified CD4:IgG_1 hinge fusion protein per mouse. From one group, blood samples were collected by retro-orbital bleeds 5 min, 30 min, 6 hr, and 48 hr after injection; from the other, samples were collected 10 min, 1 hr, and 24 hr after injection. For the monkey experiment, two cynomolgus monkeys with a body weight of 1.4 kg were injected in the saphenous vein with a bolus of 5 mg of purified fusion protein. One-milliliter blood samples were collected from the femoral vein at the same time intervals as for the mouse experiments. Serum concentrations of fusion protein were measured by a specific capture ELISA. Serum samples were incubated in microtiter wells coated with anti-CD4 antibody BMA041 (Behringwerke). The wells were washed, and developed with an alkaline phosphatase coupled antibody to human IgG (Zymed). The data for all animals are pooled in the figure shown.

DISCUSSION

Chimeric proteins bearing the extracellular domains of cell-surface proteins fused to immunoglobulin constant domains show promise as therapeutic agents. One of the most important issues confronting these agents is the extent of autoimmune damage arising from interaction of the fusion protein with its native ligand. Fortunately, the affinity of CD4 for its presumptive natural ligand, HLA class II antigen, is very low, and the affinity for HIV envelope is quite high. We find here, using a mouse model, that the fusion molecules have low toxicity in animals, and do not interfere with the humoral response to soluble antigen *in vivo*, consistent with the expectations raised by *in vitro* studies.

High-level expression of immunoglobulin fusion proteins was obtained following either transient or stable expression in nonlymphoid cells. These observations confirm and extend previous reports of immunoglobulin expression in transfected cells (Cattaneo and Neuberger, 1987; De Waele *et al.*, 1988), and show that high-molecular-weight IgM-like complexes can be formed in the absence of the J chain. Previous studies of IgM expression by nonlymphoid transfectants have shown that absence of the J chain allows hexameric IgMs to accumulate, rather than the nearly exclusively pentameric chains found *in vivo* (Cattaneo and Neuberger, 1987). The presence of either IgG or IgM CH1 domains correlated with markedly reduced fusion expression, a result not entirely unexpected, given that CH1 is normally found associated with either a light-chain constant region or heavy-chain binding protein during the course of synthesis in B cells (Haas and Meo, 1988).

The purified fusion proteins potently inhibited virus infection and dissemination *in vitro*, and cultures treated with both fusion protein and complement fared better than cultures treated with either fusion protein alone or complement alone. In contrast, neither the IgG_1 nor the IgM fusion proteins showed significant complement-mediated cytotoxicity in a short-term chromium release assay. These results suggest that short-term assays may not measure relatively subtle or slow-acting effects that play an important role in long-term cultures, or that complement may participate in virus restriction by a mechanism divorced from cytolysis, *e.g.*, through interaction with specific receptors.

The inability to demonstrate a rapid direct cytolysis, despite high-affinity interaction and apparently normal binding of initial complement components (M. Leineweber and B. Seed, unpublished), is not unexpected. Even neutralizing antisera to HIV envelope determinants rarely show direct cytolytic activity in short-term *in vitro* assays (Nara *et al.*, 1987). Further, unlike most murine retroviruses, human retroviruses HIV-1 and HTLV-1 are little affected by exposure to human serum (Hoshino *et al.*, 1984; Banapour *et al.*, 1986). In a similar study (Capon *et al.*, 1989), it has been reported that a CD4:human IgG_1 CH1 fusion protein did not bind complement component C1q, and bound only to the high-affinity Fc receptors of the U937 cell line, which bears approximately twice as many low-affinity as high-affinity receptors (Vaughn *et al.*, 1985; Looney *et al.*, 1986; Fanger *et al.*, 1989). The reasons for these discrepancies are not known, but may be related to the presence of the CH1 domain, Traunecker *et al.* (1989), for example, found that a CD4:mouse IgG_{2a} hinge fusion protein binds C1q. Further detailed analysis of the fusion proteins in HIV/SIV *in vitro* and *in vivo* models (see, for example, Watanabe *et al.*, 1989) will provide data about whether these molecules represent a realistic approach to AIDS therapy.

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APPENDIX 3

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–

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 coincubation 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 (NH_2 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

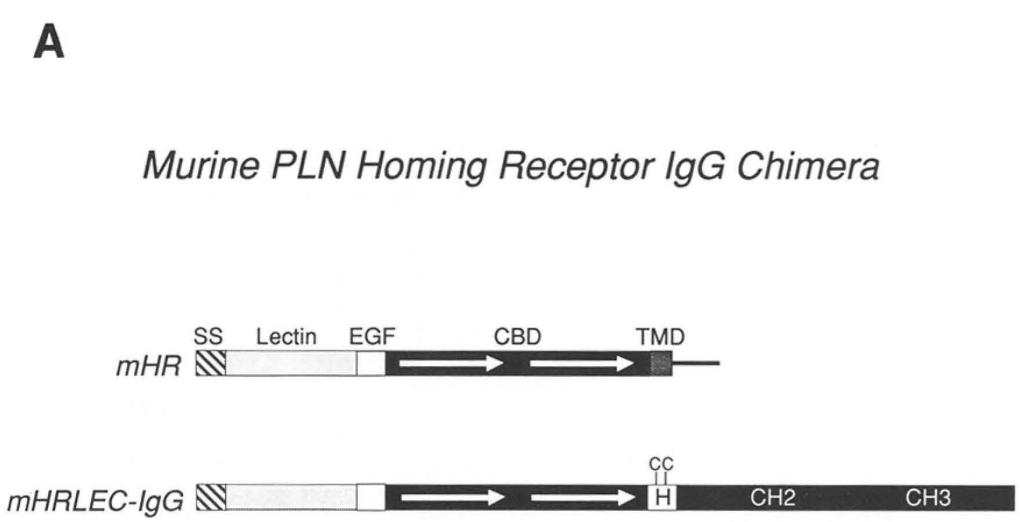
The ability of the mHR-IgG chimera to recognize the yeast polyphosphomannan (PPME), was analyzed in an ELISA format as described (19). Briefly, purified mHR-IgG chimera in Dulbecco's PBS was coated onto Immulon-2 microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4°C. Nonspecific 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_2O_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%



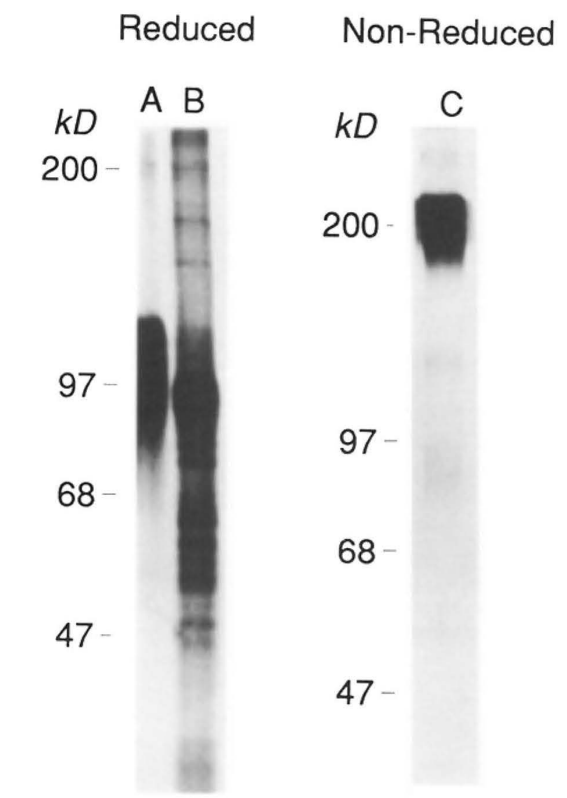


Figure 1. Construction and expression of a mHR-IgG chimera containing the lectin-egf complement binding motifs. (*A*) The protein domains of the murine homing receptor (mHR) are shown: NH_2-terminal signal sequence (*SS*), lectin, egf, and duplicated complement binding domains (*CBD*), transmembrane 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.

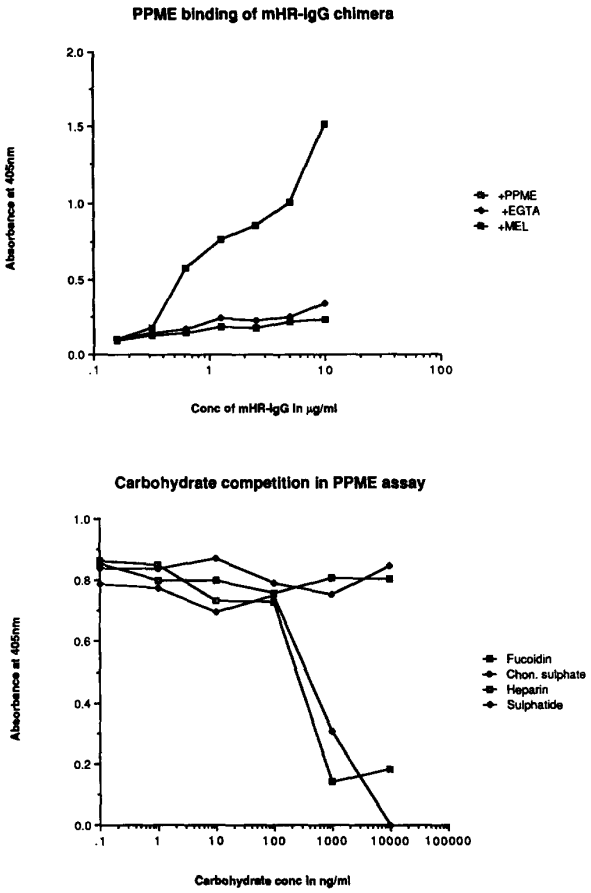


Figure 2. Binding of mHR-IgG to PPME. Purified mHR-IgG was quantified using an ELISA assay as described in Materials and Methods. The purified chimera was coated onto microtiter wells and reacted with PPME, after which the bound PPME was detected with a polyclonal antibody in conjunction with alkaline 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

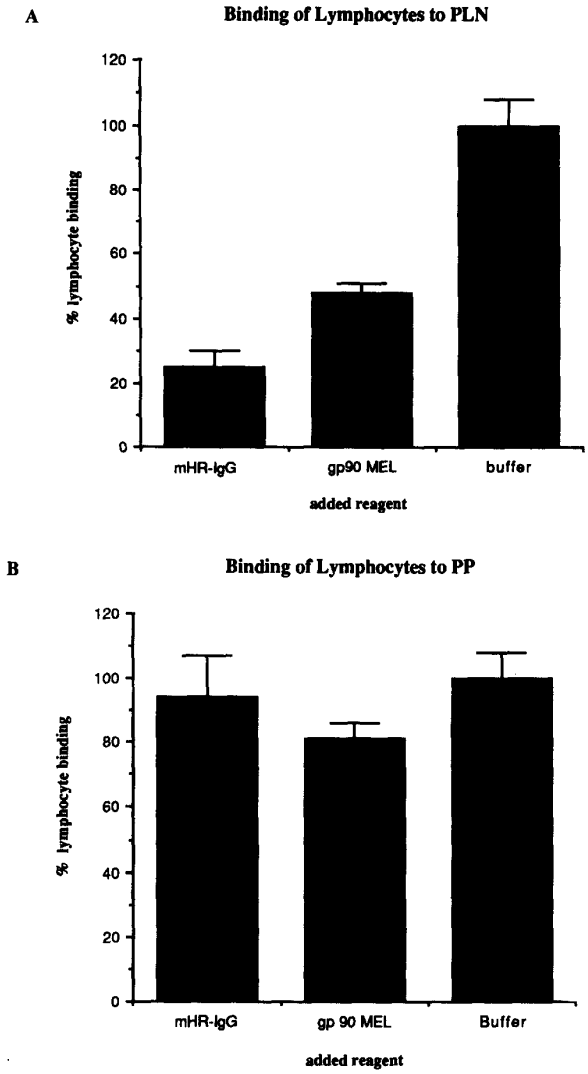


Figure 3. Inhibition of lymphocyte attachment to pln HEV by mHR-IgG chimera and by gp90^{MEL}. Cryostat sections of pln and pp were pretreated with buffer alone, purified mHR-IgG chimera or spleen-derived gp90^{MEL}, both at 1 μg/section. After the sections were washed, lymphocytes, that had been preincubated 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 (~1 μg/section), when prereacted with pln tissue sections, inhibited lymphocyte attachment to HEV by ~75%. Spleen-derived gp90^{MEL} (~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

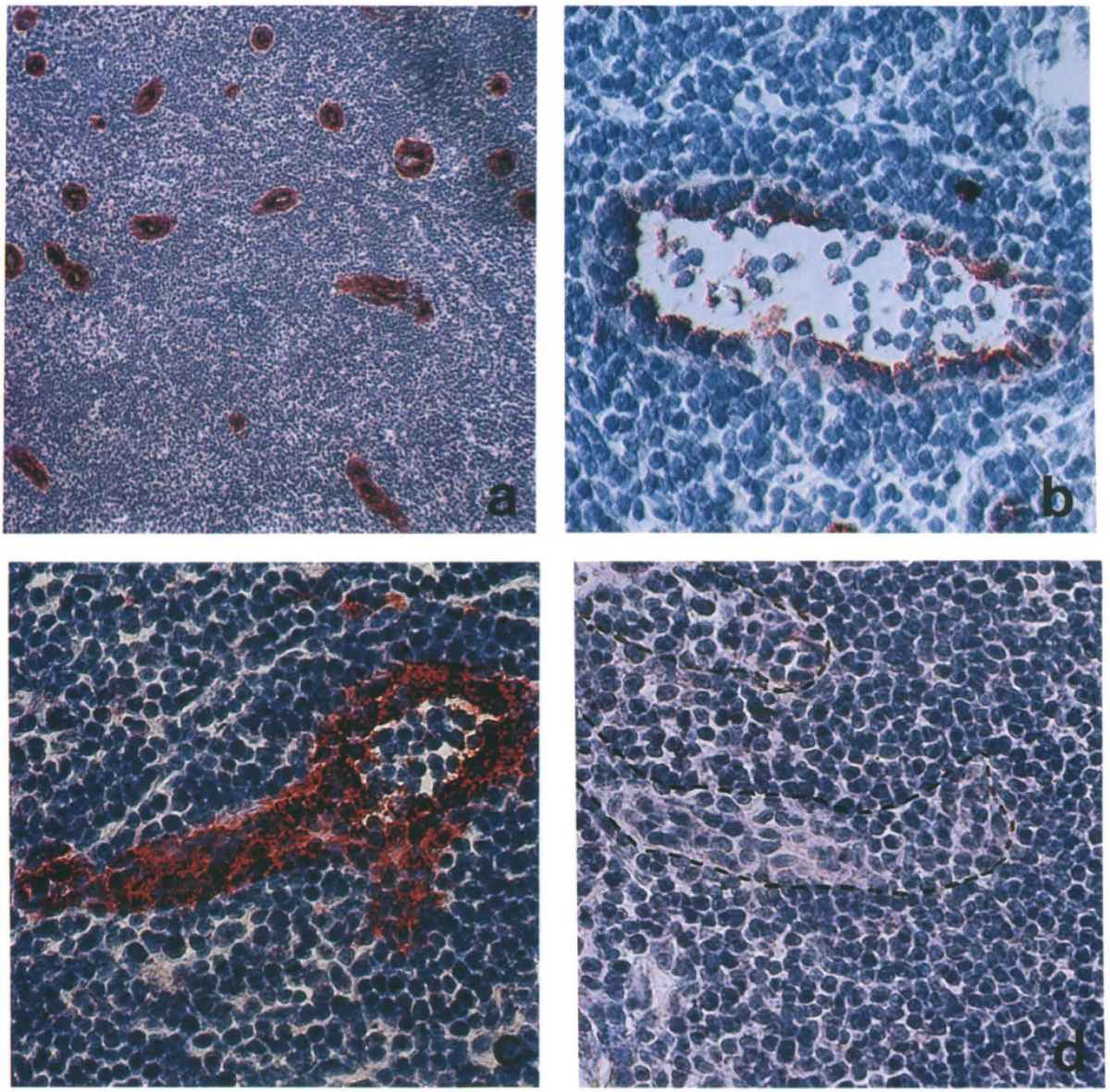


Figure 4. Histochemical staining of HEV with the mHR–IgG chimera. Cryostat-cut sections of a mouse pln and a pp were reacted with the chimera (3 μg of chimera/section, concentrated culture medium supernatant) and processed for HRP histochemistry as described in Materials and Methods. (*a*) Staining of 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 interaction 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-

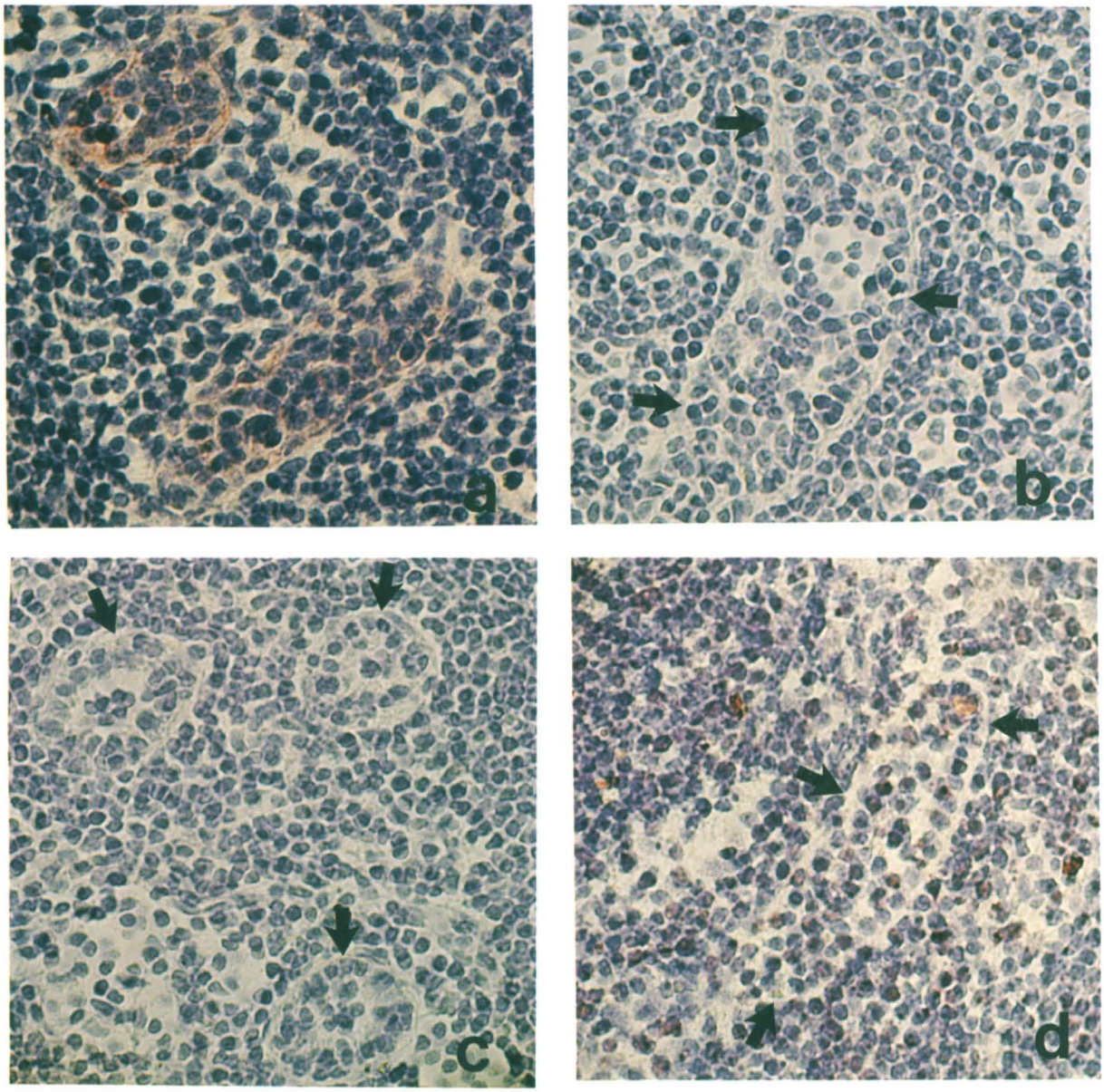


Figure 5. Inhibition of mHR-IgG chimera staining of pln HEV. Sections of pln were stained with 1 μg/section of purified chimera under four conditions. (*a*) Control, no inhibitors present. Two stained HEV are evident, the top one in cross-section and the bottom one in longitudinal section. The staining 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 inflammatory 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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APPENDIX 4

Declaration of Lynne Weaver

I, Lynne Weaver, declare the following based on my personal knowledge:

1. I have been employed by Randolph College (formerly Randolph-Macon Woman's College) since 1989, and I currently hold the title of Serials Coordinator and Library Accounts at the Lipscomb Library. My duties include the coordination and management of parts of Lipscomb Library's collection, including the acquisition and cataloging of serial publications such as journals and other periodicals. I have worked at the library with serial publications since 1994. As such, I am familiar with the procedures of the Lipscomb Library for processing serial publications, such as journals, including the acquisition, receiving, stamping, cataloging and indexing, and shelving of these publications. In addition, as part of my general duties, I am familiar with the process of searching for and locating documents in the Lipscomb Library.

2. I was asked to locate a physical copy of the June 1990 issue of *The Journal of Cell Biology* (Vol. 110, No. 6) which contained the article "A Homing Receptor-IgG Chimera as Probe for Adhesive Ligands of Lymph Node High Endothelial Venules" by Watson et al. A true and correct scanned copy of the cover of the journal issue, introductory pages of the issue, and the requested article, obtained as explained below, is attached as Appendix A to this declaration.

3. In my experience, journals such as *The Journal of Cell Biology* typically are searchable in the index of a library. In searching the index of Lipscomb Library, a copy of the requested issue of *The Journal of Cell Biology* was found in the online catalog, which indicated that a copy of the desired issue was available in the Lipscomb Library. A search of the library's holdings then located the requested issue in the place it was expected to be found as indicated in the catalog.

4. The requested issue of *The Journal of Cell Biology* was in the condition I expected to find it. On viewing the issue, I found no reason to suspect that it had been altered or was otherwise inauthentic. One of the introductory pages of the issue bears a stamp from the Lipscomb Library (and also bearing Randolph College's then-name Randolph-Macon Woman's College) dated June 14, 1990.

5. After determining that I had located the requested issue of *The Journal of Cell Biology*, I scanned the cover of the issue, the introductory pages including the masthead and the library stamped page, and the requested article by Watson, as shown in Exhibit A.

6. The scanned journal issue and article in Exhibit A are unaltered from how I found them, including the Lipscomb Library's date of receipt stamp, which bears the date June 14, 1990.

7. As my duties include the receipt, processing, and indexing of serial publications, including journals such as *The Journal of Cell Biology*, I am personally aware of the procedures used by Lipscomb Library for such publications since 1994. Although there may be technical differences in how such publications were processed in 1990, to the level of detail discussed below, I believe the procedures would have been the same in 1990, as they have essentially been the same since I began working with serial publications at Lipscomb Library in 1994. Additionally, I was working at Lipscomb Library in other capacities when this issue was acquired and processed by Lipscomb Library, and I am unaware of any change in procedures that occurred during the 1989-1994 time period.

8. Journals such as *The Journal of Cell Biology* are now, and would have been in 1990, sent to a wide range of subscribers upon publication, including libraries. As such, the receipt of such a journal by Lipscomb Library would indicate that it also was received by a number of subscribing institutions and individuals around the same time.

9. Lipscomb Library, upon receipt of such a journal, would stamp the journal with the date of receipt, and then typically would have the journal indexed and shelved either on the day of receipt or within one business day of receipt.

10. The stamping of a serial publication typically would occur on either the date of receipt or within one business day of receipt of the publication. Such stamping was regularly done at Lipscomb Library for all received serial publications.

11. As such, the stamp on the June 1990 issue of *The Journal of Cell Biology* bearing the date June 14, 1990, indicates that the issue was received by the library on or shortly before June 14, 1990, and that soon after the issue was stamped with the date, it was indexed and shelved as part of the ordinary course of business of the library. At that point, the issue would have been accessible to interested library patrons.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code. I also understand that any willful false statement may jeopardize the results of the proceedings in which this declaration is submitted.

Respectfully Submitted,

June 30, 2017
Date

Lynne Weaver
Lynne Weaver

EXHIBIT A

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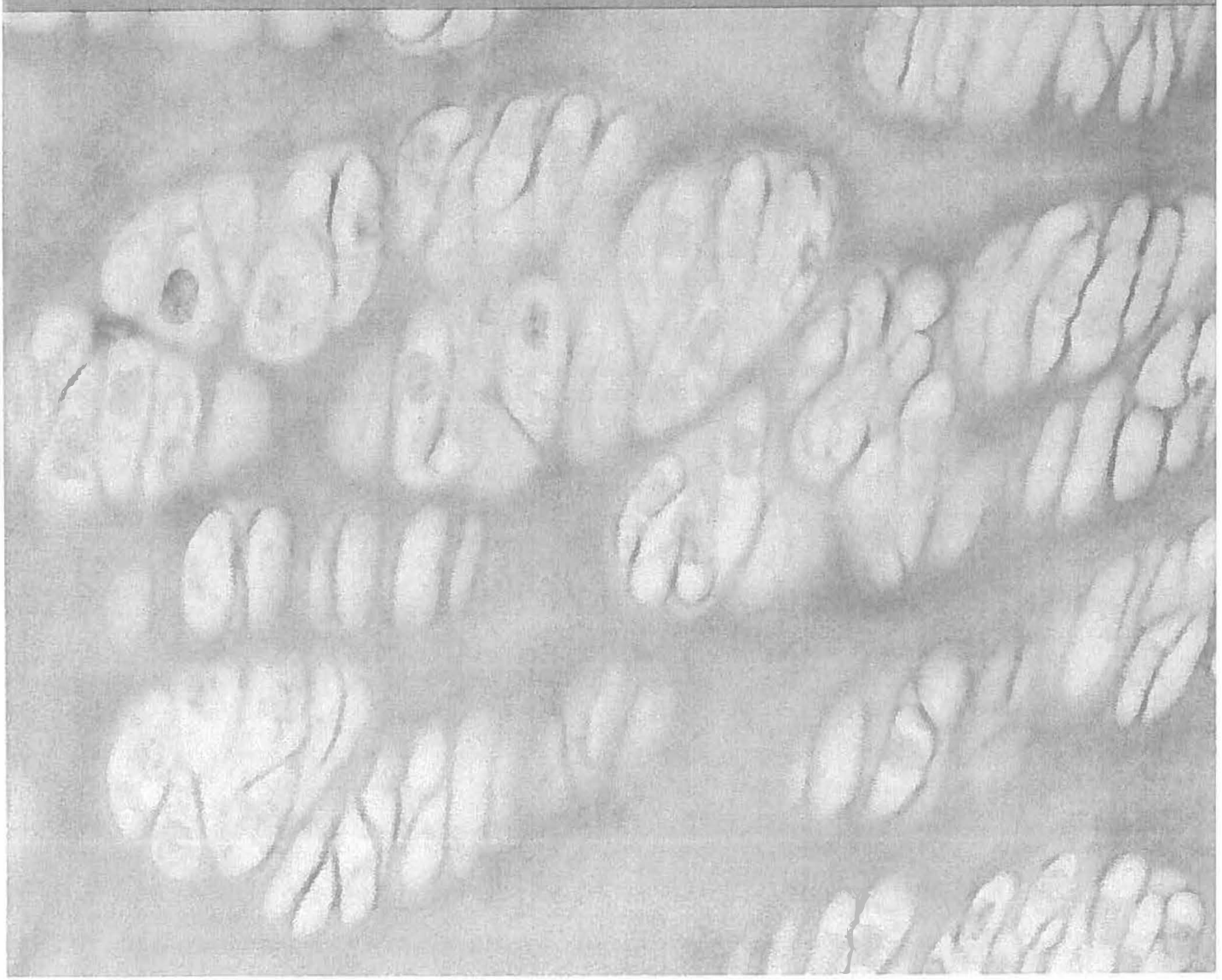

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A Homing Receptor–IgG Chimera as a Probe for Adhesive Ligands of Lymph Node High Endothelial Venules

Susan R. Watson,* Yasuyuki Imai,‡ Christopher Fennie,* Joyce S. Geoffroy,‡ Steven D. Rosen,‡ and Laurence A. Lasky*

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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^{MFL}) 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^{MFL} 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–

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 coincubation 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 (NH_2 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_2O_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%

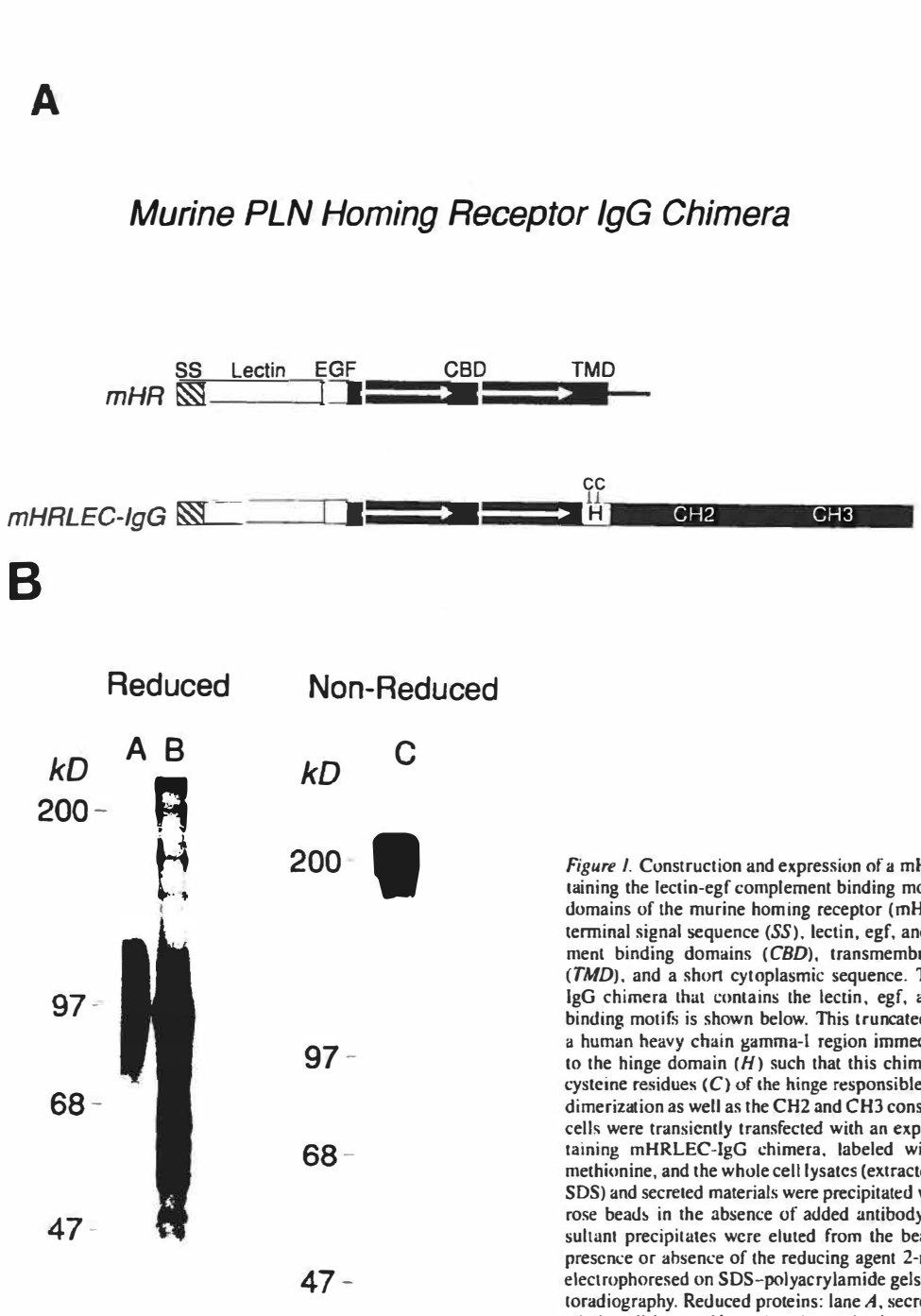

Figure 1. Construction and expression of a mHR-IgG chimera containing the lectin-egf complement binding motifs. (*A*) The protein domains of the murine homing receptor (mHR) are shown: NH_2-terminal signal sequence (*SS*), lectin, egf, and duplicated complement binding domains (*CBD*), transmembrane 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.

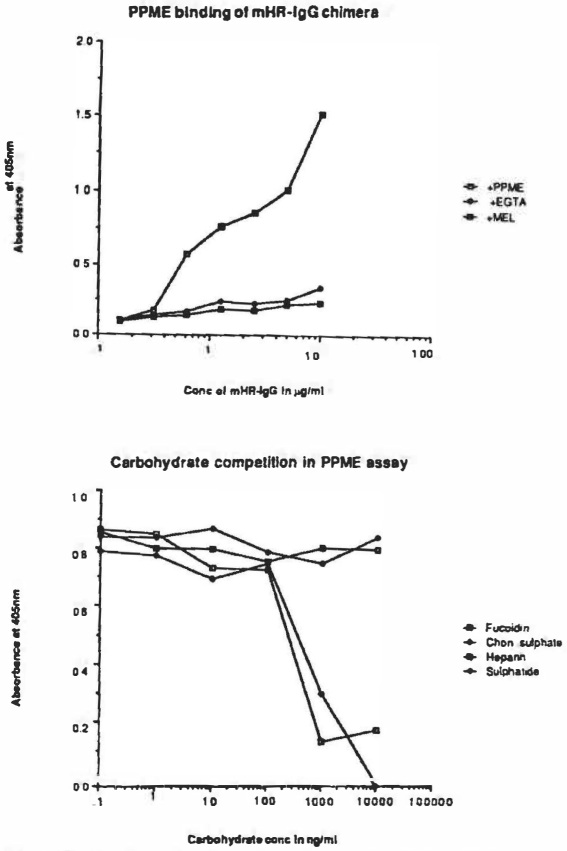

Figure 2. Binding of mHR-IgG to PPME. Purified mHR-IgG was quantified using an ELISA assay as described in Materials and Methods. The purified chimera was coated onto microtiter wells and reacted with PPME, after which the bound PPME was detected with a polyclonal antibody in conjunction with alkaline 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 avi[illegible] of the interaction between the receptor and its ligand on [illegible] endothelial surface. Finally, we felt that inclusion of the Ig[illegible] 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

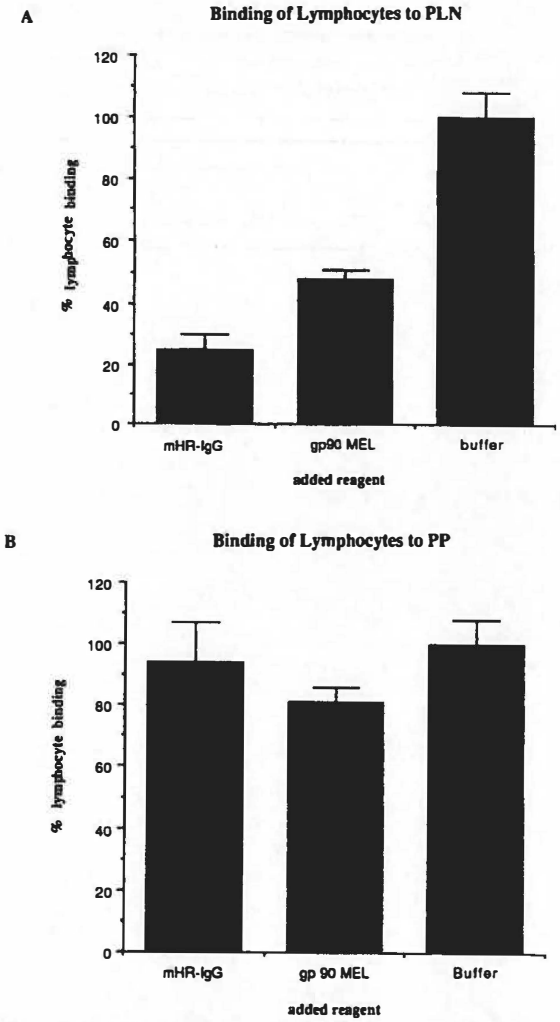

Figure 3. Inhibition of lymphocyte attachment to pln HEV by mHR-IgG chimera and by gp90^{MEL}. Cryostat sections of pln and pp were pretreated with buffer alone, purified mHR-IgG chimera or spleen-derived gp90^{MEL}, both at 1 μg/section. After the sections were washed, lymphocytes, that had been preincubated 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 (~1 μg/section), when prereacted with pln tissue sections, inhibited lymphocyte attachment to HEV by ~75%. Spleen-derived gp90^{MEL} (~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

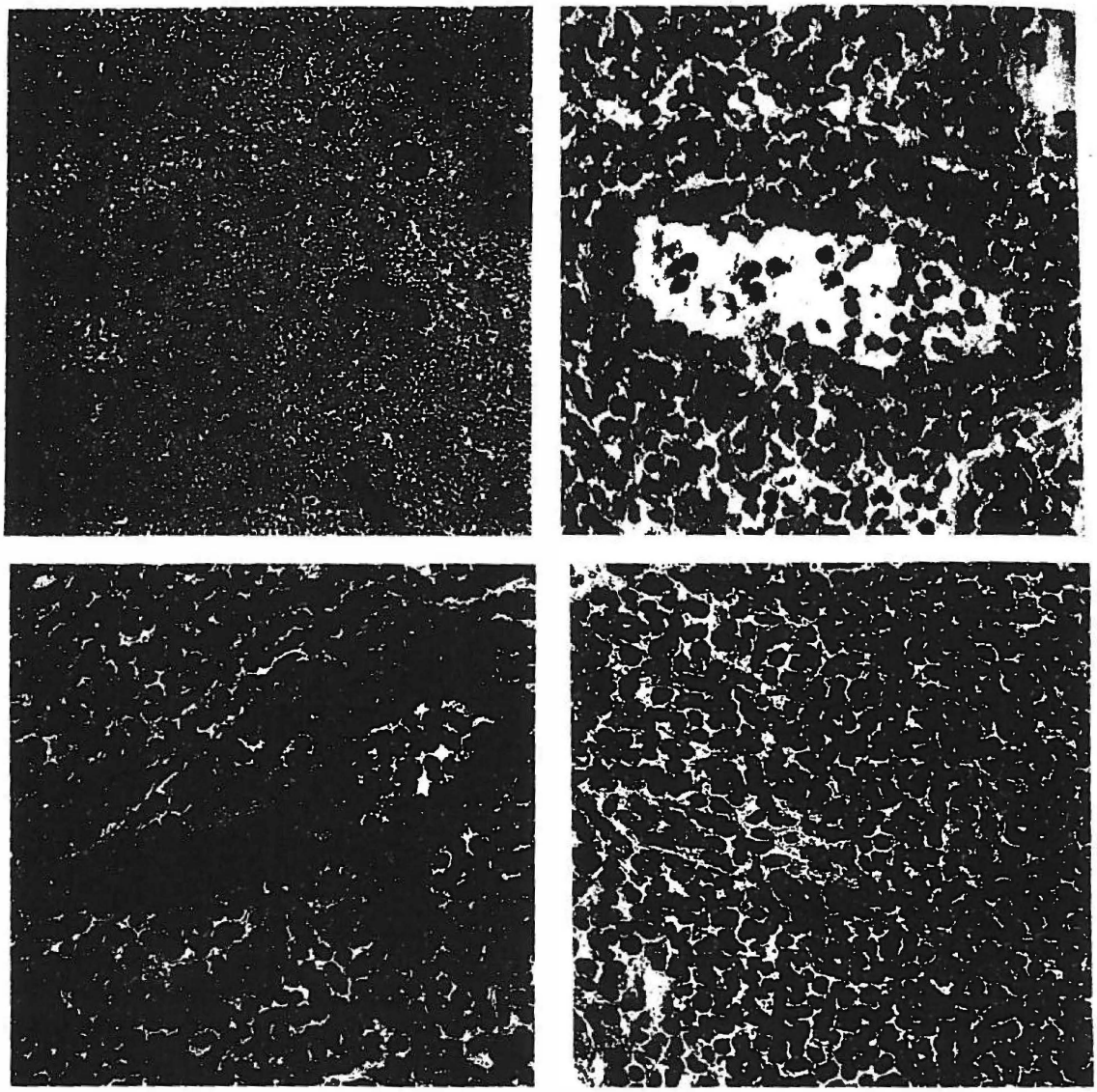

Figure 4. Histochemical staining of HEV with the mHR-IgG chimera. Cryostat-cut sections of a mouse pln and a pp were reacted with the chimera (3 μg of chimera/section, concentrated culture medium supernatant) and processed for HRP histochemistry as described in Materials and Methods. (*a*) Staining of 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 interaction 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-

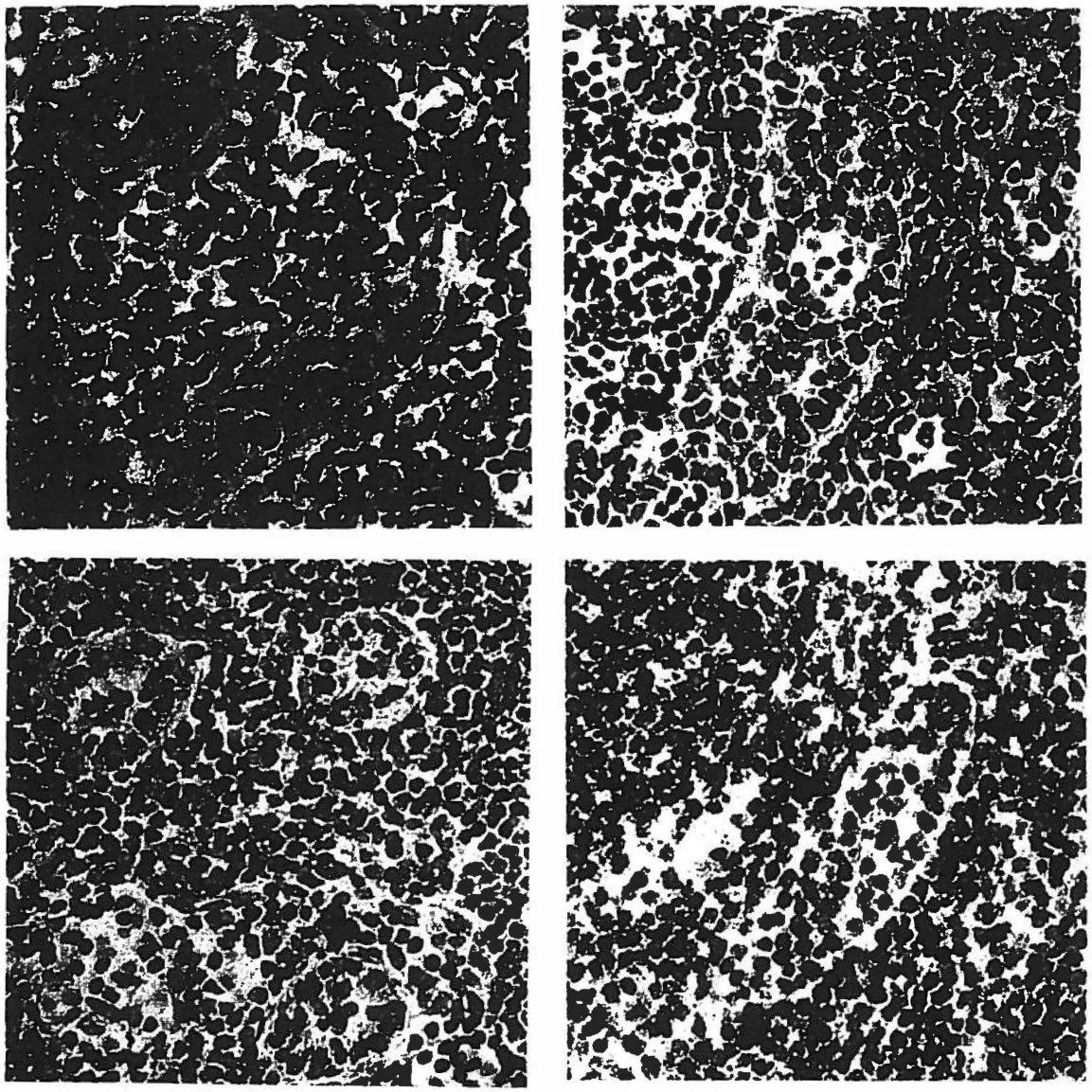

Figure 5. Inhibition of mHR-IgG chimera staining of pln HEV. Sections of pln were stained with 1 μg/section of purified chimera under four conditions. (*a*) Control, no inhibitors present. Two stained HEV are evident, the top one in cross-section and the bottom one in longitudinal section. The staining 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 inflammatory 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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