IDENTIFICATION OF A WIDELY DISTRIBUTED 90-kDa GLYCOPROTEIN THAT IS HOMOLOGOUS TO THE HERMES-1 HUMAN LYMPHOCYTE HOMING RECEPTOR

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Homing of recirculating lymphocytes from the blood into the lymphoid tissues is mediated by 90-kDa homing receptors on the lymphocyte cell surface, allowing selective binding to specialized endothelial lining high endothelial venules. This study describes two novel mAb, NKI-P1 and NKI-P2, directed against functional epitopes of a human lymphocyte homing receptor, gp90. Biochemical studies demonstrated that these antibodies recognize a 90-kDa glycoprotein which is similar to the Ag recognized by the mAb Hermes-1. This notion was confirmed by immunohistochemical studies showing identical reaction patterns. Furthermore, it was observed that NKI-P1 and NKI-P2 blocked adhesion of lymphocytes to high endothelial venules. Immunohistochemical, immunofluorescence, and immunoprecipitation studies revealed that gp90 is widely expressed on hemopoietic cells including lymphocytes, macrophages/dendritic cells, myeloid cells, and erythrocytes. The gp90 is also expressed on a number of nonhemopoietic cells such as endothelial cells, certain epithelial cells, and fibroblasts. In addition to its expression on normal cells, gp90 is present on a spectrum of tumor cell lines of lymphoid, monocytic, epithelial, glial, and melanocytic origin. In addition to the 90-kDa product, the antibodies immunoprecipitate several polypeptides in the range of 120 to 200 kDa. Interestingly, it was observed that certain mamma tumor cell-line cells lack the 90-kDa polypeptide indicating the heterogeneous expression of the molecules recognized by these antibodies. These results indicate that the 90-kDa glycoprotein homologues of the Hermes-1 human lymphocyte homing receptor are expressed on hemopoietic tissues as well as on a number of nonhemopoietic tissues and tumor cell lines. Although the function of these molecules in nonlymphoid cells is presently unknown, they might play a role in cell-cell or cell-matrix adhesion.

Cell-adhesion molecules are thought to play a critical role in many cellular processes such as growth regulation, cell migration, and organogenesis. In the immune system, interactions via Ag specific receptors are required for the induction and regulation of the immune response and for effector cell function. Cell-adhesion molecules enhance the efficacy of these specific T lymphocyte accessory and T lymphocyte target cell interactions (1, 2). Moreover, they are important for a variety of other cell-cell and cell-substrate interactions in the immune system (1–6). Recent studies with mAb that interfere with adhesive cell functions have defined a number of these cell-surface molecules. They include the Ag of the LFA-1 family, consisting of LFA-1, the C3bi receptor, and the p150,95 Ag (1–3); the LFA-3 and CD2 Ag (1, 2); the ICAM-1 Ag (2, 7); and the Hermes-1 LHR (5, 8).

The recent identification of different functional domains on the α-chain of LFA-1 (9) and C3bi receptor (10) prompted us to attempt to raise new mAb to as yet unidentified epitopes on these molecules. To this end mice were immunized intrasplenically with immunoprecipitated LFA-1 family Ag. Here we describe two mAb, NKI-P1 and NKI-P2, that were obtained by using the above protocol. Interestingly, these mAb both immunoprecipitated a major product with an m.w. of 90,000 (gp90), which is distinct from the β-chain of LFA-1 but proved homologous to the Hermes-1 LHR. This Ag is widely distributed on cells of both hemopoietic and non-hemopoietic origin.

MATERIALS AND METHODS

Preparation of mAb. BALB/c mice were immunized intrasplenically with immunoprecipitated LFA-1 family Ag. The immunoprecipitates were obtained from lysates of human monocytes (>95% pure as detected by staining for nonspecific esterase) obtained by centrifugal elutriation (11). Immunoprecipitations were carried out with the anticommon β-chain antibody CLB-LFA1/1 (12) and thus yielded all three LFA-1 family Ag. Four days after the third immunization, spleen cells were fused with SP2/0 myeloma cells and hybridoma supernatants were screened for reactivity with human monocytes by indirect immunofluorescence. In addition supernatants were tested for their effect on JY cell aggregation. Two positively reacting hybridomas were cloned fourfold by limiting dilution and were designated NKI-P1 (lgG1) and NKI-P2 (lgG1). The mAb SPV-L7 (lgG1), SPV-L10 (lgG1), NKI-L15 (lgG2a) specific for the LFA-1α subunit were raised in our laboratory (13). Furthermore we used CLB-LFA-1/1 (lgG1) specific for the β subunit of LFA-1 (12), W6/32 (lgG2a) specific for a nonpolymorphic determinant of HLA-ABC (14) and 15D9 (IgGl) specific for T200 (Dr. R. van Lier, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Hermes-1 (rat IgG2a), which reacts with the Hermes-1 human LHR*

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3 Abbreviations used in this paper: HEV, high endothelial venule; LFA-1, leukocyte function associated antigen-1; LHR, lymphocyte homing receptor; JY, Epstein-Barr virus transformed B cell.
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(15), was kindly provided by Dr. Butcher (Department of Pathology, Stanford University, Stanford, CA).

Aggregation assay. JY cells were seeded in 96-well flatbottom microtiter plates (Cladon, Costar, Cambridge, MA). Each well contained 1.5 x 10^5 cells, previously washed and resuspended in Iscove's medium containing 10% FCS. The mAb were added (1000-fold dilution of ascites) and the plates were stored for various time periods at 37°C. The percentage of aggregated cells was determined as described (9).

Peptide mapping, immunoprecipitation, and SDS-PAGE analysis.

Two methods were used for cell surface radiolabelling, the iodogen method or the lactoperoxidase method. For immunoprecipitation, labeled cells were lysed at 4°C (1 h) with 1% Nonidet P-40 in 0.01 M triethanol amine-HCl, pH 7.8, 0.15 M NaCl. 1 mM phenylmethylsulfonyl fluoride, 0.62 mg/ml ovomucoid trypsin inhibitor (Sigma, St. Louis, MO). After removal of insoluble material, the lysate was preclarified successively with mouse IgG coupled to protein A sepharose and protein A sepharose itself. The preclarified lysates were incubated with specific mAb, coupled, and crosslinked to protein A sepharose for 2 to 3 h at 4°C. The immunoprecipitates were washed with lysis buffer containing 0.5% Nonidet P-40 (Sigma). SDS-PAGE was carried out according to the Laemmli procedure (16). Kodak SAR film was used in combination with Intensifier screens for autoradiography.

Peptide mapping using S. aureus V8 protease. Polypeptide gel bands were cut out of a fixed and dried gel and swollen in 0.125 M Tris-HCl buffer (pH 6.8), 0.1% SDS, 1 mM EDTA. The slices were loaded onto a second polyacrylamide gel and overlaid with Tris buffer containing 10% glycerol and 1 µg V8 protease (ICN, Plainview, NY). Electrophoresis was stopped for 30 min when the dye front reached the beginning of the running gel. After this incubation period, which was performed at room temperature, electrophoresis was continued.

Immunoperoxidase staining. Staining was performed on acetone-fixed cryostat sections by an indirect immunoperoxidase procedure. The serial frozen sections were incubated with NKI-P1 or Hermes-1, fixed cryostat sections by an indirect immunoperoxidase procedure.

RESULTS

Hybridomas were produced from mice immunized intraperitoneally with immunoprecipitated LFA-1 alpha chain. Selection on reactivity with human monocytes by immunofluorescence yielded two hybridomas, NKI-P1 and NKI-P2.

NKI-P1 and NKI-P2 stimulate homotypic aggregation of JY cells. Inasmuch as mAb against functional epitopes of the LFA-1 alpha and beta chain inhibit both the spontaneous and PMA-induced homotypic interactions of JY cells, we studied the possible inhibitory effect of supernatants from NKI-P1 and NKI-P2 on this interaction. Surprisingly, we found that mAb secreted by the hybridomas NKI-P1 and NKI-P2 did not inhibit but, in contrast, stimulated the aggregation of the LFA-1 positive cell line JY (Table I). As expected mAb against LFA-1 alpha or the common beta subunit strongly inhibited the aggregation (Table I). A control mAb against HLA class-1, which is abundantly expressed by JY cells, did not affect aggregation (Table I).

NKI-P1 and NKI-P2 immunoprecipitate a major product with an m.w. of 90,000 (gp90) distinct from the beta chain of LFA-1. The mAb secreted by NKI-P1 and NKI-P2 immunoprecipitated from monocytes a major product with an m.w. of 90,000 (gp90), and variable amounts of 120, 150, and 180 kDa products (Fig. 1, lane A and B). In all of the immunoprecipitations carried out, the 90-kDa band was the most prominent by far. These products are not disulfide-linked because the same pattern is observed under reducing and nonreducing conditions, although the individual bands migrate slightly faster under nonreducing conditions, indicating that some intramolecular disulfide bonds are present. The 95-kDa beta-subunit of LFA-1 (Fig. 1, lane C and D) was clearly distinct from gp90 in that the latter molecule migrates as a broad band with an m.w. slightly lower than LFA-1 alpha (Fig. 1).

gp90 is homologous to the Hermes-1 LHR. Unexpectedly we observed that the immunohistochemical staining pattern of sections of human lymphoid tissues with the mAb NKI-P1 and NKI-P2 was identical to the pattern obtained with Hermes-1, a recently described mAb directed against a 90-kDa Ag involved in the binding of human lymphocytes to HEV (data not shown). To further investigate the possibility that NKI-P1 and NKI-P2 recognized the Hermes-1 LHR, we tested the capacity of these mAb to interfere with the in vitro binding of human PBL to HEV. As shown in Table II, both mAb inhibit binding of lymphocytes to peripheral lymphnode HEV. Inhibition occurs at the level of the lymphocyte, since preincubation of the HEV with the mAb did not cause inhibition of binding.

Comparative immunoprecipitation studies with the mAb Hermes-1, NKI-P1 and NKI-P2 confirmed the notion that these mAb recognize similar Ag. Like NKI-P1 and NKI-P2, Hermes-1 was also found to immunoprecipitate a major band of 90 kDa and several additional bands with a much lower intensity at approximately 120, 150,
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Figure 1. SDS-PAGE analysis of NKI-P1 and NKI-P2 immunoprecipitates from a 125I-labeled lysate of human monocytes. Human monocytes were obtained by centrifugal elutriation and directly surface-labeled by the lactoperoxidase method. After solubilization (1% NP-40) and preclearing, the lysate was used for immunoprecipitation with NKI-P2 (lane A), NKI-P1 (lane B), SPV-L10 (lane C), SPV-L7 (lane D), normal mouse IgG (lane E). Analysis was performed by SDS-PAGE under reducing conditions on a continuous gradient gel (5 to 12.5%).

Figure 2. SDS-PAGE analysis of NKI-P1, NKI-P2 and Hermes-1 immunoprecipitates from a 125I-labeled lysate of human monocytes. Monocytes were radiolabeled by the iodogen method and the lysate was divided into equal parts for immunoprecipitation with normal mouse IgG (lane A), NKI-P1 (lane B), Hermes-1 (lane C) and NKI-P2 (lane D). The immunoprecipitates were analyzed on a continuous gradient gel of 6 to 8 under reducing conditions.

TABLE II
Inhibition of adhesion of peripheral blood lymphocytes to HEV by NKI-P1 and NKI-P2

<table>
<thead>
<tr>
<th>mAb (Ag)</th>
<th>% Inhibition of lymphocyte binding to HEV (X ± SE)*</th>
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<tbody>
<tr>
<td></td>
<td>mAb added to lymphocytes</td>
</tr>
<tr>
<td>15D9 (T200)</td>
<td>-2 ± 6</td>
</tr>
<tr>
<td>NKI-P1 (gp90)</td>
<td>51 ± 6</td>
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<tr>
<td>NKI-P2 (gp90)</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>SPV-L7 (LFA-1α)</td>
<td>59 ± 5</td>
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* Mean ± SE of three independent experiments.

and 180 kDa from a lysate of human monocytes (Fig. 2). Moreover, one dimensional peptide mapping of the 90 kDa products with V8 protease resulted in similar proteolytic fragments (Fig. 3A, lane A, B, and C) that, as expected, are clearly distinct from those of LFA-β (Fig. 3B, lane D and E). Additional evidence was obtained from sequential immunoprecipitation studies. Preclearing of a lysate with the Hermes-1 antibody and vice versa resulted in a strong reduction (>95%) of the immunoprecipitated material obtained with mAb NKI-P1 or NKI-P2 (data not shown). From these data we conclude that gp90 is homologous to the so-called LHR and distinct from the β-chain of LFA-1. Crossblocking studies with NKI-P1, NKI-P2, and Hermes-1 showed that the former two mAb recognize overlapping epitopes on the gp90 molecule distinct from the epitope recognized by Hermes-1 (data not shown).

gp90 shows a broad tissue distribution. The cell and tissue distribution of gp90 was studied using NKI-P1, NKI-P2, and Hermes-1 mAb in immunohistochemical, immunofluorescence, and immunoprecipitation studies. The immunohistochemical studies were performed on
frozen tissue sections of normal human organs. gp90 was found to be expressed in the cytoplasm and on cell membranes of a variety of cells in lymphoid and nonlymphoid tissues (Table III). In lymphoid tissues, a high expression of the gp90 Ag was observed on most cells of both the B and T cell lineage as well as on macrophages, on interdigitating and follicular dendritic cells, and on vascular endothelium. In addition, the mucosal epithelium of the tonsil stained positive. In accordance with previous reports on the expression of the Hermes-1 LHR on normal lymphoid cells (5, 15), most germinal center cells and cortical thymocytes which are nonrecirculating lymphocytes were gp90 low or negative. In most of the nonlymphoid tissues tested, we observed staining of fibroblasts, endothelial cells, and macrophage-like cells, including the alveolar macrophages of the lung and Kupffer cells of the liver. Staining of epithelial cells was consistently found in the mucosa of tonsils, the epidermis, the salivary gland, and the pancreas, whereas intestinal epithelium, hepatocytes, bile duct epithelium, pancreatic acinar cells, and A375 malignant melanoma cell lines (Fig. 4).

To exclude the possibility that the apparently broad tissue distribution of gp90 was due to cross-reaction with shared epitope(s) on unrelated molecule(s), comparative immunoprecipitation studies using a panel of cell lines were performed. The results shown in Figure 5 demonstrate that the major band with an m.w. of approximately 90,000 could be precipitated from most cell lines tested. Interestingly, we observed a marked heterogeneity in m.w. and the amount of the various polypeptides immunoprecipitated from the different cell lines. For example, the mamma-carcinoma cell lines T47D, MCF-7, and ZR-75 (Fig. 5B, lanes 3, 4, and 5) lack the 90-kDa molecule but express several higher m.w. polypeptides. From these data we conclude that the molecules recognized by NKI-P1 and NKI-P2 are identical to the Hermes-1 LHR and are heterogeneously expressed by many cells of both hemopoietic and nonhemopoietic origin.

Discussion

A central step in lymphocyte recirculation and homing is the migration of lymphocytes from the blood into the surrounding tissues via the wall of HEV (5, 18, 19). This migration is thought to involve tissue specific adhesion of lymphocytes to the endothelial lining of HEV via LHR expressed on the lymphocyte cell surface (5, 6, 8, 15, 17). The important role of LHR in lymphocyte homing was initially inferred from functional studies of lymphocyte migration in vivo (20–22) and of lymphocyte-HEV binding in vitro (5, 17, 20–23). More recently, the mAb Mel-14 described by Gallatin et al. (24) and the mAb Hermes-1/3 (6, 15) described by Jalkanen et al. have defined a set of 90-kDa murine and human LHR glycoproteins.

We found that the gp90 molecule identified by the novel mAb, NKI-P1 and NKI-P2, is homologous to the human Hermes-1 LHR and distinct from the β-chain of LFA-1. This conclusion is based on immunoprecipitation and peptide mapping studies comparing the gp90 band precipitated by NKI-P1 and NKI-P2 with that obtained with anti-LFA-1 β and Hermes-1. Although we used human monocytes in these initial experiments, NKI-P1 and NKI-P2 also recognize a functional epitope on the LHR as it is expressed on lymphocytes. This is evident from our tissue distribution studies showing identical staining patterns for NKI-P1, NKI-P2, and Hermes-1 as well as from the functional studies demonstrating inhibition of lymphocyte adhesion to HEV by NKI-P1 and NKI-P2. The capacity of NKI-P1 and NKI-P2 to inhibit binding of lymphocytes to HEV indicates that these antibodies recognize an epitope on the LHR distinct from Hermes-1 since the latter antibody is noninhibitory (15). This conclusion is confirmed by our crossblocking studies.

As is shown by the immunohistochemical, immunofluorescence, and immunoprecipitation studies, we observed that the gp90 molecule recognized by NKI-P1 and NKI-P2 (but also by Hermes-1) is rather widely distributed among both hemopoietic and nonhemopoietic cells, and in this respect there is some resemblance to ICAM-1, a recently described cell-adhesion molecule and counterstructure of LFA-1 (2, 7, 25). Although this molecule has approximately the same m.w., tissue distribution and biochemical studies showed that it is clearly distinct from gp90 (our unpublished observations). Interestingly, however, it has recently become clear that the Hermes-1 LHR, Pgp-1, and the extracellular matrix receptor type III (ECMR III) might all be similar to CD44. Mackay et al. showed that Pgp-1 has an m.w. of approximately 94,000 and is homologous to CD44 (26). Wayner, Carter, and Gallatin (27) observed that the 90-kDa phosphorylated transmembrane molecule ECMR III binds collagen and is homologous to the Hermes-1 LHR. Data presented in the CD44 workshop recently held at the 4th International Conference on human leukocyte differentiation antigens (Vienna, February 21–25, 1989) supported the notion that these Ag are related and may all fall within the CD44 cluster.

The Ag recognized by NKI-P1, NKI-P2, and Hermes-1 are also expressed on a spectrum of nonhemopoietic tumor cell lines including bladdercarcinoma, ovariumcarcinoma, mammacarcinoma, gliblastoma, and melanoma.
cell lines and display a consistent m.w. heterogeneity in these different cell types. This expression on tumor cell lines cannot be attributed to culture conditions inasmuch as expression was also observed in several native tumors (our unpublished observation). It will be important to determine if the gp90 and related higher m.w. molecules on nonhemopoietic cells can also mediate cell adhesion to endothelium and/or play a role in other adhesive cell-cell and cell-matrix interactions. If so, the Hermes-1 LHR and related molecules on nonlymphoid cells would constitute a novel family of adhesive glycoproteins. As was recently described for LHR expression in non-Hodgkin's lymphoma in mouse (28) and man (29), expression of these molecules on nonhemopoietic tumor cells might also be related to their metastatic potential. In this context, it is of interest that certain nonlymphoid murine tumors show tissue-specific adhesion to capillary endothelium in vitro (30), and correspondingly, preferential

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**Figure 4.** Expression of LHR and LFA-1 on various human cell-line cells, monocytes and erythrocytes. The tumor cell-line cells (A375, M14, OVCAR-4, T24, U87MG) were treated with EDTA to obtain single-cell suspensions. After washing, these cells, U937, monocytes and erythrocytes were incubated with mAb (ascites in a 500-fold dilution) followed by incubation with FITC conjugated goat anti-mouse IgG F(ab)2. Fluorescence was measured with a FACS. As negative control antibody, an anti-idiotyp antibody (S6) (IgG1) was used.

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**Figure 5.** SDS-PAGE analysis of NKI-P1 and NKI-P2 immunoprecipitates from various cell line lysates. Cells were labeled with $^{125}$I using the lactoperoxidase method. Single cell suspensions of the adherent cell lines, were obtained after treatment with EDTA. The immunoprecipitates were analyzed under reducing conditions on a 5 to 15% gradient polyacrylamide gel. A, lane 1, M14 melanoma; lane 2, OVCAR-4 ovarian carcinoma; lane 3, T24 bladder carcinoma. B, lane 1, A375 melanoma; lane 2, HBL100 normal human mammary cell line; lane 3, T47D mammary carcinoma; lane 4, MCF-7 mammary carcinoma; lane 5, ZR-75 mammary carcinoma.
metastasis in vivo (31).

All three mAb immunoprecipitate small amounts of 120, 150, and 180 kDa glycoproteins in addition to gp90. A similar observation was recently reported by Jalkanen et al., who described a subset of Ag recognized by Hermes-1 and -3 with a molecular mass of 180 to 200 kDa (32).

Interestingly, we found that all three, but not the normal mammary gland cell line HBL100, lacked expression of the major 90 kDa polypeptide present in all other cell lines tested. Pulse-chase experiments of Jalkanen et al. indicated that a precursor of 76 kDa appears to be processed to a 90 kDa and a 180 to 200 kDa form, the latter by addition of chondroitin sulfate side chains. The additional bands of 120 and 150 kDa (Fig. 2) identified in our experiments might represent molecules with less chondroitin side chains because Jalkanen et al. observed similar bands after incomplete digestion of the 180 to 200 kDa band with chondroitinase-ABC. It should be noted that different cell types were used in our biochemical studies and that the differences observed may reflect m.w. heterogeneity of the high m.w. glycoproteins similar to that of the major 90 kDa band. Thus far it is not clear whether the absence of the 90 kDa polypeptide observed in the mammary carcinoma cell-line cells must be ascribed to a lack of gp90 biosynthesis, or to an almost complete conversion of the 90 kDa polypeptide to the higher m.w. forms. Biosynthesis experiments are underway to clarify this observation.

A still unresolved question is the biochemical basis of organ-specific lymphocyte HEV binding. Minor modifications of the LHR adhesion receptors or their ligands might convey tissue specificity. Alternatively, tissue specificity could be the net result of multiple adhesion-molecule interactions. Thus, several types of molecules have been described which are involved in the adhesion of lymphocytes to endothelium. Among these are LFA-1 (6, 33) and VLA-4 (34), both members of the integrin superfamily. Furthermore, it has become clear that Elam-1 and Mel-14, which mediate leukocyte-endothelial cell interactions, are closely related adhesion proteins (35, 36).

Finally, the fact that NKI-P1 and NKI-P2 were raised through immunization with immunoprecipitated LFA-1 family molecules needs comment. This result could be explained by the possibility that these mAb recognize an epitope shared by gp90 and LFA-1. Alternatively, it could indicate that gp90 and LFA-1 were somehow associated in the immunoprecipitate used for immunization. Although at present we cannot definitively discriminate between either of these two possibilities, our observation that NKI-P1 and NKI-P2 sometimes coprecipitate small amounts of LFA-1 (unpublished observation), as well as the demonstration that LFA-1 is also involved in lymphocyte adhesion to HEV, might favor the latter explanation.

Additional studies are required to clarify this point.

In conclusion, our data demonstrate that gp90 is homologous to the Hermes-1 LHR and that this glycoprotein is expressed on hemopoitetic as well as on a number of nonhemopoietic tissues and cell lines. Although the function of these molecules is at present incompletely understood, they might represent a novel family of adhesion molecules involved in (tissue specific) cell-cell and cell-matrix interactions.

Note added in proof. During processing of this paper it was reported that indeed CD44 is homologous to Pgpr-1 and the human Hermes-1 LHR (37). Furthermore, cloning of the gene encoding the Hermes-1 LHR showed that this molecule is related to the cartilage proteoglycan core and link proteins (38, 39).

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