



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 16/28, 2/00, C12N 5/20, 1/00, G01N 33/53, A61K 39/395	A1	(11) International Publication Number: WO 95/31482 (43) International Publication Date: 23 November 1995 (23.11.95)
(21) International Application Number: PCT/US95/05921 (22) International Filing Date: 11 May 1995 (11.05.95) (30) Priority Data: 08/243,288 17 May 1994 (17.05.94) US (71) Applicant: LUDWIG INSTITUTE FOR CANCER RE- SEARCH [CH/US]; 1345 Avenue of the Americas, New York, NY 10105 (US). (72) Inventors: SANZ-MONCASI, Maria, Pilar; Independencia 14, 3 ^o I, E-50004 Zaragoza (ES). GARIN-CHESA, Pilar; Amriswilstrasse 7, D-88400 Biberach (DE). STOCKERT, Elisabeth; 1275 York Avenue, New York, NY 10021 (US). OLD, Lloyd, J.; 1345 Avenue of the Americas, New York, NY 10105 (US). RETTIG, Wolfgang, J.; Amriswilstrasse 7, D-88400 Biberach (DE). (74) Agent: HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10022 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: MONOCLONAL ANTIBODY SPECIFIC FOR VASCULAR ENDOTHELIAL CELL ANTIGEN ENDOGLYX-1, THE ANTIGEN ITSELF, AND USES THEREOF (57) Abstract An antigen which is specific to vascular endothelium is described. Described is a cell surface antigen which consists of four subunits, of 190, 145, 125 and 110 kd as determined by SDS-PAGE under reducing conditions as is a monoclonal antibody specific to this antigen. Uses, both diagnostic and therapeutic, are also described.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5 **MONOCLONAL ANTIBODY SPECIFIC FOR VASCULAR ENDOTHELIAL**
 CELL ANTIGEN ENDOGLYX-1, THE ANTIGEN ITSELF,
 AND USES THEREOF

 This application is based upon work supported, in part,
by a grant from the National Cancer Institute (CA-57486). The
10 U.S. government may have certain rights in the invention.

FIELD OF THE INVENTION

 This invention relates to the study of the surface of
vascular endothelial cells. More particularly, it relates to
the characterization of a surface antigen specific to vascular
15 endothelial cells, a monoclonal antibody which specifically
binds to the antigen, and a hybridoma which produce the
monoclonal antibody. The antigen, referred to hereafter as
endoGlyx-1, is a cell surface glycoprotein consisting of four
subunits. More details of the antigen are provided in the
20 application which follows. Also described are various uses
for the monoclonal antibody, such as diagnostic and
therapeutic agents, as well as for the antigen itself, as,
e.g., an immunogen.

BACKGROUND AND PRIOR ART

25 Interest in surface antigens of vascular endothelial
cells has come from several lines of research, including
studies of lymphocyte homing, inflammation, blood clotting,
and tumor metastasis. Exemplary of the vast body of
literature in this area are Bevilacqua, Ann. Rev. Immunol. 11:
30 767-804 (1993); Shimizu et al., Immunol. Today 13: 106-112
(1992); Zimmerman et al., Immunol. Today 13: 93-99 (1993);
Butcher et al., Cell 67: 1033-1037 (1991); Schlingemann et
al., Lab. Invest. 52: 71-76 (1985); Schlingemann et al., Am.
J. Pathol. 138: 1335-1347 (1991); Picker et al., Ann. Rev.
35 Immunol. 10: 561-591 (1992); Asborn, Cell 62: 3-6 (1990);
Roth, Immunol. Today 13: 100-105 (1992); Weidner et al., New
Eng. J. Med. 324: 1-8 (1991). Throughout these studies,
monoclonal antibodies (mAbs) have proven to be valuable tools
for dissecting the antigenic structure of endothelial cells in
40 different organs, tissues or segments of the vascular system,
and the endothelial responses to inflammation, tissue damage,

5 and tumor growth. Furthermore, mAbs have been used in the biochemical and molecular genetic characterization of endothelial antigens and in the functional analyses of endothelial molecules *in vitro* and *in vivo*.

10 Several categories of endothelial antigens have been distinguished, based on their distribution patterns in normal and lesional blood vessels. These include (i) antigens with wide distribution in the vascular system, such as Factor VIII-related antigen (FVIIIIRA), PAL-E, and CD13, as per Schlingemann et al., Lab Invest. 52: 71-76 (1985); Kuzu et al., J. Clin. Path. 45: 143-148 (1992); (ii) antigens
15 restricted to vessels in specific organs or tissues, or to unique histologic types of vessels, as illustrated by vascular addressins and GlyCAM-1; and (iii) inducible antigens, such as E-selectin, VCAM-1, and ICAM-1, that are not present or
20 expressed at low levels in normal endothelium but are upregulated in inflamed tissues *in vivo* and/or induced or cultured endothelial cells by proinflammatory cytokines, notably tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Pober et al., Lab. Invest. 64: 301-305 (1991); Kuzu et al.,
25 Lab. Invest. 69: 322-328 (1993)).

However, none of the previously identified endothelial antigens serves as a unique endothelial cell lineage marker; i.e., they are either not widely expressed in the vascular system (antigenic heterogeneity) or they are expressed on
30 certain non-endothelial cells. Time and again, when mAbs with apparent specificity for endothelium were subjected to rigorous specificity analyses, including tests with panels of cultured cells that represent diverse cell lineages, and immunohistochemical analysis of fetal and adult normal tissues
35 and lesional tissues, unexpected reactivities and nonendothelial cells were uncovered. Bevilacqua, supra; Shimizu et al., supra; Zimmerman et al., supra; Butcher et al., supra, Kuzu et al., supra; Ruiter et al., J. Invest. Dermatol. 93: 25-32 (1989); Gougos et al., Int. Immunol. 4:
40 83-92 (1992); Alles et al., J. Histochem. Cytochem. 34: 209-214 (1986). For example, FVIIIIRA is present in megakaryocytes

5 and platelets, and CD31, CD36, and CD34 are expressed by
subsets of lymphocytes and myelomonocytic cells. Similarly,
BW200 is expressed in mesothelium and glomerular epithelium,
PAL-E is detected in the epidermis (Ruiter et al., supra); and
10 endoglin is present in placental trophoblastic cells,
hematopoietic precursors, and several other cell types (Gougos
et al., supra).

A cell surface antigen specific to vascular endothelium,
referred to as endoGlyx-1 hereafter, has now been identified.
This molecule is a glycoprotein with four subunits, bound
15 together by disulfide bonds. The molecule appears to have a
molecular weight in excess of 500 kd on SDS-PAGE under non-
reducing conditions. Its four subunits, under reducing
conditions, appear to have molecular weights of about 190 kd,
140 kd, 125 kd, and 110 kd. The antigen is described herein,
20 as is a monoclonal antibody which specifically binds to it.
Also disclosed are various diagnostic and therapeutic uses for
the monoclonal antibody and the antigen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a number of panels showing
25 immunohistochemical staining and detection of endoGlyx-1 in
normal and malignant tissues. In figures 1A and C-M, the
magnification is X25; in figure 1B it is 12.5. Specifically:

Figure 1A: newborn umbilical cord vein (endoGlyx-1⁺);

Figure 1B: normal adult breast (endoGlyx-1⁺
30 capillaries);

Figure 1C: breast carcinoma (abundant endoGlyx-1⁺
capillaries surrounding a tumor cluster);

Figure 1D: 16 week fetal kidney (endoGlyx-1⁺
endothelial cells);

Figure 1E: 16 week fetal lung (endoGlyx-1⁺
35 endothelial cells);

Figure 1F: fetal thymus (16 weeks), (endoGlyx-1⁺
endothelial cells);

Figure 1G: Hemangioma with endoGlyx-1⁺ endothelial
40 cells;

Figure 1H: Hemangioma of 1G, stained with mAb PAL-E,

- 5 showing only focal immunostaining;
- Figure 1J: Angiosarcoma with endoGlyx-1⁺ neoplastic cells;
- Figure 1K: Ovarian cancer with tumor cells in an endoGlyx-1⁺ blood vessel;
- 10 Figure 1L: Osteoarthritis with endoGlyx-1⁺ endothelial cells, and endoGlyx-1⁺ inflammatory cells;
- Figure 1M: The osteoarthritis of 1L, stained with anti- α CD31, showing immunostaining of
- 15 endothelial cells and infiltrating lymphocytes.

Figures 2A and 2B show the immunoprecipitation of endoGlyx-1 glycoproteins from NP40 extracts of [³H] glucosamine (figure 2A), or Trans ³⁵S (figure 2B) labelled

20 HUVEC cultures. Lysates were tested with mAb H572 ("+"), or unrelated, negative control IgG1 ("-"). The immunoprecipitates were separated on 6% SDS-gels under reducing conditions, and were detected by fluorography.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

The first step of the study involved the preparation of monoclonal antibodies ("mAbs" hereafter), which bound, specifically, to endothelial cells.

BALB/C mice were immunized with cultured human umbilical vein endothelial cells ("HUVEC" hereafter). These cells are described by Jaffe et al., J. Clin. Invest. 52: 2745-2753 (1973); Jaffe et al., J. Biol. Chem. 262: 8557-8565 (1987); both of which are incorporated by reference in their entirety. The mice were immunized with 10⁶ cells which had been

30 suspended in 100 μ l of PBS. The first immunization was subcutaneous, followed by intraperitoneal immunization, also with 10⁶ cells. Two further immunizations were carried out, at four week intervals. Each immunization was performed subcutaneously, with 10⁶ cells in PBS. No adjuvant was used

35 in any of the immunizations.

40

Spleens of the subject animals were removed, and

5 separated into individual cells which were then fused to SP2/0 myeloma cells.

Following fusion and culturing, the culture supernatants from the hybridoma were tested for binding to a panel of cultured cell types. The panel consisted of HUVECs, EAhy 926
10 which is an HUVEC derived immortalized cell line (Edgell et al., Proc. Natl. Acad. Sci. USA 80: 3734-3737 (1983)), fibroblasts, breast cancer cell line MCF-7, renal cancer cell line SK-RC-7, and melanoma cell line SK-MEL-28. The initial screening, described herein, was carried out using the mixed
15 hemadsorption rosetting assay, as described by Rettig et al., J. Immunol. 183: 4884-4489 (1987), incorporated by reference herein. Any supernatants which showed selective reactivity with HUVEC were selected for further analysis. Fifteen hybrid cultures were identified in this fashion, and these hybridomas
20 were subcloned, using standard limiting dilution methods.

The fifteen cloned hybridoma cells were then expanded in vitro, and the culture supernatants were tested for reactivity using a larger panel of cultured cell lines. Supernatant from one clone, i.e., H572, was found to contain a monoclonal
25 antibody which bound exclusively with HUVECs. The mAb was unreactive with cultured fibroblast, sarcoma, neuroblastoma, melanoma, glioma and carcinoma cells. It was also unreactive with cells taken from peripheral blood and bone marrow, including lymphocytes, granulocytes, monocytes,
30 megakaryocytes, platelets, and erythrocytes. Table 1 sets forth the results. The mAb, while reactive with HUVECs, was unreactive with cultured cells of mesenchymal, neuroectodermal, and epithelial derivation. "FACS" analysis of peripheral blood cells, and immunocytochemical analysis of
35 nucleated bone cells showed that H572 is unreactive with normal lymphoid and hematopoietic cells. Again, the assay employed was that of Rettig et al., supra.

5 Table 1. Cell surface reactivity of mAb H572 with short-term cultures
 of normal human cells, established tumor cell lines, and
 normal, uncultured peripheral blood and bone marrow cells

Cell type	Designation	mAb H572 (titer ¹)
<u>Cultured cells:</u>		
HUVEC (n-12)		1,250-6,250
Fibroblast	GM05387, WI-38, Hs27, F135-35-18	-
Sarcoma	SW872, TE-85, SAOS-2	-
15 Neuroblastoma	LA-N-1, SK-N-SH, SMS-MSN	-
Melanoma	SK-MEL-13, SK-MEL-28, SK-MEL-31	-
Glioma	SK-MG-12, SK-MG-26, U251MG	-
Carcinoma	MCF-7, MDA-MB468, SK-RC-28	-
	HT-29, DLD-1, SK-OV-6	-
<u>Peripheral blood and bone marrow:</u>		
Lymphocytes		-
Granulocytes		-
25 Monocytes		-
Megakaryocytes, platelets		-
Erythrocytes		-

30 Example 2

 H572 was then tested for *in vivo* reactivity with tissues,
 in immuno-histochemical assays. Tissue samples were obtained
 either from autopsy or surgical specimens. These samples were
 embedded in OCT, frozen in isopentane which had been precooled
 with liquid nitrogen, and stored at -70°C. The tissues were
 tested using the well known avidin-biotin immunoperoxidase
 method, as described by Garin-Chesa et al., J. Histochem.
 Cytochem. 36: 383-389 (1988), the disclosure of which is
 incorporated by reference in its entirety, but is outlined
 herein. Briefly, five micron thick frozen sections were cut,
 mounted on poly-(L-lysine)-coated slides, air-dried, and fixed
 in acetone (4°C, ten minutes). The sections were treated with

5 0.3% H₂O₂ for three minutes to block endogenous peroxidase, followed by blocking with normal horse or goat serum for 30 minutes, at room temperature.

10 The slides were incubated at 4°C, with mAbs (hybridoma culture supernatant, dilution of 1:2; or purified Ig, 5-20 ug/ml), rabbit anti-FVIIIIRA (diluted 1:120,000), or negative control antibodies, for 12-18 hours. Sections were washed, and incubated with biotinylated horse anti-mouse or goat anti-rabbit Ig, for 30 minutes at room temperature, followed by
15 avidin-biotin horseradish peroxidase complex (1:100 dilution, at 1:1 ratio). Final reaction product was visualized with the chromogen 3,3'-diaminobenzidine. Sections were then counterstained with hematoxylin. This extensive analysis included umbilical cord, skin, mammary gland, lung, esophagus, stomach, small and large intestine, liver, pancreas, thyroid
20 gland, adrenal gland, kidney, urinary bladder, prostate, testis, ovary, fallopian tube, uterus, smooth and skeletal muscle, thymus, spleen, and lymph node tissues. The mAb was found to react exclusively with vascular endothelial cells. Exemplary of the staining pattern with normal cells are
25 figures 1A and 1B. EndoGlyx-1 expression was found in capillaries, veins, arterioles, and muscular arteries. Staining was consistent with uniform cell surface, and cytoplasmic distribution of the antigen, with some accentuation of the abluminal side of endothelial cells in
30 some vessels, as per figure 1A.

35 Organ specific patterns of endoGlyx-1 expression were observed. In the liver, endothelial cells of vessels in portal spaces were positive, central veins were weakly positive, and sinusoids were negative. In the spleen, trabecular veins and arteries, and central arterioles of the white pulp were positive, but sinusoidal endothelial cells of highly vascularized red pulp of the spleen were negative. In lymph nodes, subcapsular sinus endothelial cells, afferent and efferent blood vessels showed immunoreactivity.

40 In lungs, all vessels showed expression, including alveolar capillaries.

5 In kidney tissues, endoGlyx-1 staining was seen in glomerular tufts, with variable staining intensity, and in peritubular capillaries, and larger arterial and venous vessels.

Example 3

10 Normal tissues of a 16 week old fetus were also available for the study, including skin, connective tissue with primitive cartilage formation, thymus, spleen, pancreas, liver, stomach, ovary, lung, kidney, heart, cerebral cortex and cerebellum. The assay was the same as above. In each
15 tissue, strong endoGlyx-1 immunostaining of the vascular endothelium was observed, with the exception of liver and splenic sinusoids. Figures 1D, 1E and 1F present the staining patterns observed in fetal, lung, fetal kidney, and fetal thymus. No other cell types present showed any endoGlyx-1
20 staining.

Example 4

 The same assay was carried out in vascular neoplasms. Table 2 summarizes the results. Some hemangiomas and angiosarcomas retained expression of endoglyx-1 (figures 1G
25 and 1J), while neoplastic cells of hemangiopericytomas were consistently endoGlyx-1 negative, which is in keeping with their derivation from pericytes rather than vascular endothelial cells. In contrast, nontransformed, capillary endothelial cells in the stroma of hemangiomas showed expected
30 endoGlyx-1 staining.

Example 5

 Extended analysis with mAb H572 of endoGlyx-1 expression in tumor blood vessels of over 100 neoplasms of diverse histological types, also summarized in Table 2, showed
35 consistent antigen expression in stromal blood vessels of carcinomas, sarcomas, lymphoid, and neuroectodermal neoplasms. Exemplary are the staining patterns of figures 1C and 1K, breast and ovarian cancer, respectively.

5 **Table 2.** Endoglyx-1 expression in human tumor tissues determines by the avidin-biotin immunoperoxidase method

H572 immunoreactivity				
Tumor Type	N	Tumor cells	Stromal fibroblasts	Tumor vascular endothelium
Vascular tumors:				
15 Hemangioma	3	+	-	NE
Angiosarcoma	2	+	-	NE
Hemangiopericytoma	8	-	-	+(8/8)
Other tumor types:				
Carcinomas	77	-	-	+(77/77)
20 Sarcomas	15	-	-	+(15/15)
Neuroectodermal	10	-	-	+(10/10)
Lymphomas	10	-	-	+(10/10)

+ = Antigen positive

25 - = Antigen negative

NE = not evaluatable, because of prominent immunostaining of tumor cells

Example 6

30 A comparative study was then carried out, where normal and lesional tissue were tested, side by side, with (i) mAb H572, (ii) rabbit anti-FVIII related antigen α CD31; and (iii) mAb PAL-E. The purpose was to compare specificity and sensitivity of these reagents in immunohistochemical identification of vascular endothelial cells.

35 The analysis confirmed the previous reports of variable expression of FVIII related antigen α CD31 in normal and tumor blood vessels (Kuzu et al., J. Clin. Path. 45: 143-148 (1992)). The comparison also found that the PAL-E antigen, 40 α CD31 and endoGlyx-1 are expressed widely in vascular beds of human solid neoplasms. The comparison of α CD31 distribution and endoGlyx-1, represented by figures 1L (endoGlyx-1) and 1M

5 (α CD31), shows sharp divergence. The mAbs against α CD31 tested, i.e., SG134 (Goyert et al., in McMichael et al., ed. Leukocyte Typing III. White Cell Differentiation Antigen (Oxford University Press, (1988), at 1049), and EA 195, react with endothelial cells and subsets of peripheral blood
10 leukocytes, nucleated bone marrow cells, cells on the red pulp of the spleen, and lymphoid cells present in tumor and inflammatory tissues.

 While the PAL-E antigen resembles α CD31 and endoGlyx-1 in its widespread distribution on endothelial cells, it was
15 expressed at very low levels or was undetectable in some muscular arteries and arterioles which showed strong α CD31 and endoGlyx-1 immunostaining. Also, in kidney tissue, the PAL-E antigen was present in peritubular capillaries but was absent from glomerular tufts and arterioles. Unlike α CD31 or
20 endoGlyx-1, immunostaining of the PAL-E antigen was observed in basal keratinocytes of normal epidermis, as was reported by Ruiter et al., J. Invest. Dermatol 93: 25-32 (1989). These results differ from the report of Leenstra et al., Cancer 72(1): 3061-3067 (11-15-93), wherein it was reported that the
25 PAL-E antigen was found on tumor pillories, and not on normal brain capillary pillories. Endoglyx-1 was found on both types of cells, however, showing a distinct difference in distribution. Side by side analysis of several hemangiomas for endoGlyx-1 and expression of the PAL-E antigen identified
30 some lesions uniformly positive for endoGlyx-1 expression but largely negative for the PAL-E antigen. In the latter cases, immunostaining was restricted to capillary endothelial cells in the hemangioma stroma, which will be seen by comparing figures 1G and 1H.

35 Example 7

 Radio-immunoprecipitation assays were carried out with mAb H572.

 Cultures of HUVECs were radiolabelled with a mixture of
40 [³⁵S]methionine and [³⁵S]-cysteine (40 μ Ci/ml; 1Ci=37GB1), or [³H]glucosamine (40 μ Ci/ml), by culturing for 18-24 hours in the presence of these labels, following Rettig et al., Brain

5 Res. 590: 219-228 (1992), the disclosure of which is incorporated by reference. The cells were extracted in lysis buffer [0.01M Tris-HCl/0.15M NaCl/0.01M MgCl₂/0.5% Nonidet P-40/aprotinin (20 ug/ml)/2 mM phenylmethanesulfonyl fluoride, and then used for immunoprecipitation assays, following well known techniques, using either H572, or unrelated, negative control.

Any immunoprecipitates were then separated on 6% SDS-gels, under reducing conditions, and were detected by fluorography.

15 The results, presented in figure 2, show the mAb H572 precipitated several protein species. These migrated at Mr 190,000, 140,000, 125,000, and 110,000 on SDS-PAGE, using reducing conditions.

When the experiment was done using SDS-gels under non-reducing conditions, the immunoprecipitate barely entered the 7% SDS-running gel, thereby suggesting a molecular size of greater than 500,000 for a disulphide bonded complex.

Since the 190, 140 and 125 kd subunits - and - to a lesser extent, the 110 kd subunit are all seen after [3H]glucosamine labelling, the subunits are glycoproteins.

25 The molecule will be referred to as "endoGlyx-1" hereafter.

Example 8

30 An experiment was carried out to determine if endoGlyx-1 expression is modulated by inflammatory cytokines. In metabolic labelling experiments using Trans ³⁵S label, following example 7, supra, HUVECs which were cultured in the presence of TNFα (50 ng/ml), IL-1β (2 ng/ml), or TGF-β1 (2 ng/ml), did not show changes in abundance or subunit composition of the molecule.

40 The foregoing disclosure teaches a methodology for production of monoclonal antibodies such as H572, which specifically bind to an antigen characteristic of vascular endothelial cells. The antigen is a glycoprotein, with four subunits, having molecular weights of about 190 kd, 140 kd,

5 125 kd, and 110 kd as determined by SDS-PAGE, under reducing conditions. Under non-reducing conditions, the antigenic complex exhibits a molecular weight greater than about 500 kd, as determined by SDS-PAGE.

10 Monoclonal antibodies which specifically bind to vascular endothelial cell specific antigens, such as the endoGlyx-1 molecule discussed supra, and hybridomas which produce them, are also described. Exemplary of these monoclonal antibodies is H572. A hybridoma cell line which produces H572 has been deposited at the American Type Culture Collection, 12301
15 Parklawn Drive, Rockville Maryland, under Accession Number HB 11608, on April 7, 1994, in accordance with the Budapest Treaty. This mAb is of the IgG1 subclass, although those of ordinary skill in the art will understand that the antibody class and subclass may differ in other mAbs which bind to the
20 same target.

 It is to be understood that "monoclonal antibody" as the term is used herein embraces not only the products of hybridomas, but also monoclonal antibodies produced via other well known methodologies. Such methodologies include, but are
25 not limited to, e.g., the use of cells which have been "immortalized" by transfection with Epstein Barr Virus or other immortalizing agents. Also embraced are monoclonal antibodies produced via genetic engineering, such as by transformation of appropriate host prokaryotic or eukaryotic
30 cells. Also covered are chimeric antibodies. These are well known in the art as antibodies which contain portions of antibodies from two or more species. For example, it is well known to produce chimeric antibodies which contain the CDR region of a murine monoclonal antibody, and the remaining
35 portions of a human monoclonal antibody. Such chimeras are extremely useful, e.g., in a therapeutic context. It also embraces the well known binding fragments of antibodies, such as the Fab, F(ab)₂, Fv and other binding fragments. Also covered by the invention are oligomeric, and polymeric
40 constructions, where a plurality of monoclonal antibodies of the recited specificity are complexed to each other.

5 The antibodies of the invention can clearly be used in
diagnostic methods to identify the site of vascular
endothelium or a vascular endothelium the cell, e.g., whereby
the monoclonal antibody is contacted to a sample to be
assayed, and its binding is monitored. Such binding can be
10 determined using any of the standard immunoassay protocols
known to the artisan, including, but not being limited to,
radioimmunoassays, enzyme linked immunosorbent assays,
sandwich assays, competitive assays, chromophoric assays and
so forth. Many of these assays require the use of a
15 detectable label which is attached to the antibody, and any of
the labels known to the art, including radioactive,
chromophoric, and fluorophoric substrates, enzymes, magnetic
particles, and metallic particles may be used. In carrying
out the assays, the sample of interest may be, e.g., a tissue
20 sample or body fluid sample. Further, the specificity of the
mAb permits the artisan to use it *in vivo* diagnosis. Among
the varieties of *in vivo* diagnosis which can be used,
radioimaging is particularly preferred.

 Of particular value is the use of the mAbs of the
25 invention in the identification of endothelial cell
precursors, by using an mAb in a sample which is known to be
free of endothelium cells. Binding of the antibody to target
indicates the presence of an endothelial cell precursor.
While the mAb can be used in any biological sample, such as
30 tissue samples and body fluid samples, it is especially useful
in the assaying of bone marrow and peripheral blood, where
endothelial cell precursors may be found.

 The ability of the monoclonal antibodies of the invention
to target vascular endothelium makes them particularly useful
35 in a therapeutic context.

 Normal vascular endothelial cells do not generally
proliferate in a healthy individual, in contrast to the
proliferation observed in tumor associated vascular
endothelium. By linking the monoclonal antibody of the
40 invention to a cell proliferation- inhibiting agent, one can
inhibit or retard tumor proliferation. The agent does not

5 affect those endothelial cells which are not proliferating, so
there is no problem with the complex of proliferation
inhibitor/monoclonal antibody adversely affecting other cells.
This therapy comprises administering an amount of the
monoclonal antibodies of the invention sufficient to inhibit
10 proliferation of the endothelial cells targeted, thereby
preventing angiogenesis in lesional tissue, as well as sparing
other proliferating cells, such as bone marrow, which do not
express the targeted antigen, i.e., endoglyx-1. In addition,
the mAbs may be used in connection with liposomal delivery
15 systems, where the liposome contains the inhibiting or other
therapeutic agent, and the mAbs target these to the site of
the vascular cells.

Such targeting can also be used in directed gene therapy.
This approach, carried out *in vitro*, e.g., complexes the mAbs
20 of the invention to a nucleic acid molecule which codes for a
protein of interest, or a complex which contains the nucleic
acid molecule. The nucleic acid molecule may be carried by a
liposome or other agent. As the mAb is targeted to the
endothelial cells, there is every reason to expect that the
25 gene or nucleic acid molecule of interest will also "target"
the endothelial cell.

The monoclonals, either alone or with the various
materials described supra, may be formulated in various
reagent formats. For example, the mAb can be combined with a
30 pharmacologically acceptable carrier. When used in connection
with the various materials disclosed herein, these can be
attached to the mAb to form a conjugate, the conjugate then
being combined with a pharmacologically acceptable carrier.
It is also possible to prepare a kit type of reagent, wherein
35 the mAb and the second substance are presented in separate
portions, both of which are included in a container means.
Such reagents are useful both therapeutically and
diagnostically.

Also envisioned and within the scope of the invention is
40 the isolation of both the coding region for endoglyx-1 and the
regulatory elements which control its expression. These

5 elements are of particular interest, in view of endoGlyx-1's exclusive expression by vascular endothelial cells. It is expected that such regulatory elements may be operably linked to genes of interest and then transfected into endothelial cells. Such transfection will lead to expression of the
10 protein of interest in endothelial cells only.

It has been mentioned, supra, that the mAbs of the invention are useful diagnostically, in that various labels may be attached to the mAb, with the labelled material then being used to target and/or identify endothelial cells. The
15 ability to bind specifically to these cells also places the ability to isolate or to purify endothelial cells in the hands of the skilled artisan. The basic methodology essentially involves contacting the mAbs of the invention to a sample containing the target vascular endothelial cell. The mAb has
20 been treated in some way which makes it removable from the sample. For example, the mAb can be conjugated to a solid phase, such as a column or a microbead. The mAb binds to the targeted cell, and the resulting complex is removed from the sample. The thus removed cells can be treated in various
25 ways. Genes can be inserted, and the transformants cultured for transfer back into the subject. The removed cells can also be simply cultured without transformation, so as to provide adequate supplies of the cells if and when necessary.

The isolated glycoprotein endoGlyx-1 of the invention can
30 be used as an immunogen, either alone or in combination with a suitable adjuvant. It can also be used in studies to determine its amino acid sequence, which in turns leads to the preparation of probes for isolating the nucleic acid molecule which codes for the protein of the antigen.

35 Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of
40 excluding any equivalents of the features shown and described or portions thereof, it being recognized that various

5 modifications are possible within the scope of the invention.

Claims:

1. Monoclonal antibody which specifically binds to an antigen expressed by vascular endothelial cells but not expressed by any other cell type.
2. The monoclonal antibody of claim 1, wherein said monoclonal antibody binds to a glycoprotein which consists of four subunits, said subunits having molecular weights of 190,000, 140,000, 125,000 and 110,000 as determined by SDS-PAGE.
3. The monoclonal antibody of claim 1, designated mAb H572.
4. The monoclonal antibody of claim 1, wherein said monoclonal antibody is a chimeric antibody.
5. The monoclonal antibody of claim 4, wherein said chimeric antibody consists of murine CDR regions, and the remainder of said antibody is a human antibody.
6. Antigen binding fragment of the monoclonal antibody of claim 1.
7. The binding fragment of claim 6, consisting of an Fab, F(ab)₂, or F_v fragment.
8. Hybridoma cell line which produces the monoclonal antibody of claim 1.
9. Method for identifying a vascular endothelium cell comprising contacting a sample with the monoclonal antibody of claim 1, and determining binding of said monoclonal antibody as an indication of a vascular endothelial cell in said sample.
10. The method of claim 9 wherein said monoclonal antibody is labelled.

11. The method of claim 10, wherein said monoclonal antibody is labelled with a radioactive, chromophoric, or fluorophoric label, an enzyme, a magnetic particle or a metal particle.
12. The method of claim 9, wherein said sample is a tissue sample.
13. The method of claim 9, wherein said sample is a body fluid sample.
14. Method for identifying an endothelial cell precursor in a sample comprising contacting a sample which does not contain endothelial cells with the monoclonal antibody of claim 1 and determining binding of said monoclonal antibody to a target in said sample to identify an endothelial cell precursor in said sample.
15. The method of claim 14, wherein said sample is a bone marrow sample.
16. The method of claim 14, wherein said sample is a peripheral blood sample.
17. Method for inhibiting angiogenesis in a subject comprising administering to said subject a complex of the monoclonal antibody of claim 1 and a cell proliferation inhibiting agent in an amount sufficient to prevent vascular angiogenesis by proliferating endothelial cells.
18. Method for separating a vascular endothelial cell from a sample containing said cell, comprising contacting said sample with a removable form of the monoclonal antibody of claim 1, and removing said removable monoclonal antibody following binding of said vascular endothelial cell thereto.
19. Method for delivering a desired agent to vascular

endothelium comprising administering a complex of said desired agent and the monoclonal antibody of claim 1 to a subject in need thereof, wherein said monoclonal antibody specifically delivers said desired agent to vascular endothelium.

20. Isolated, vascular endothelium associated glycoprotein consisting of four subunits having molecular weights of 190,000, 140,000, 125,000, and 110,000 as determined by SDS-PAGE.
21. Immunogenic composition comprising the glycoprotein of claim 20 and an adjuvant.

1/7

FIG. 1A

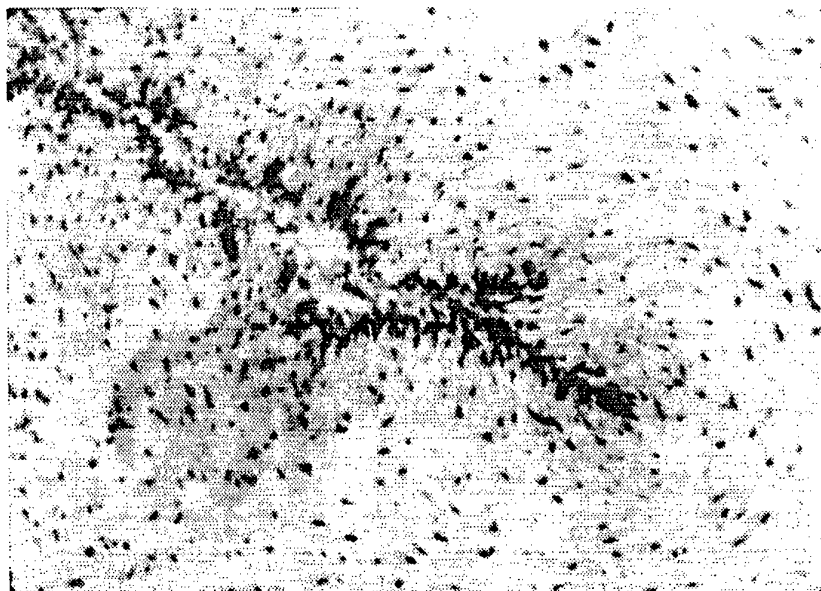


FIG. 1B

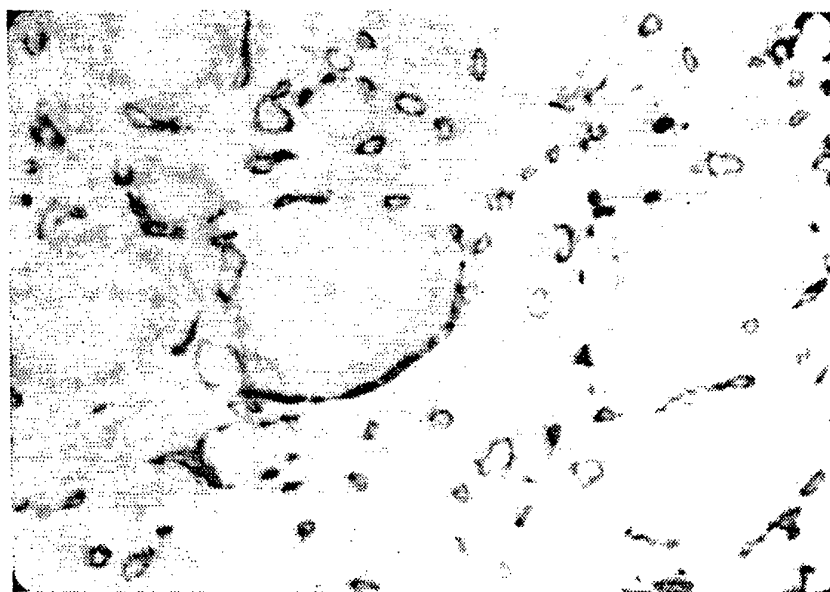


2/7

FIG. 1C



FIG. 1D



3/7

FIG. 1E

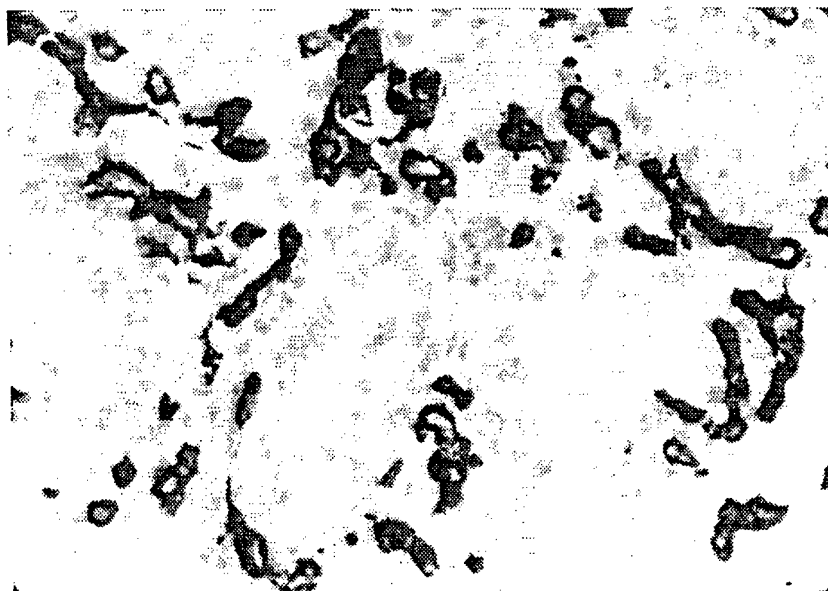
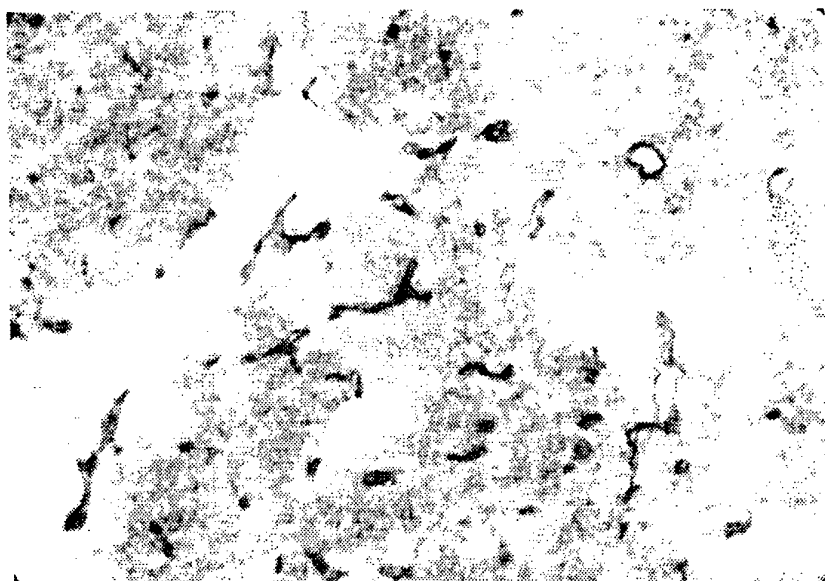


FIG. 1F

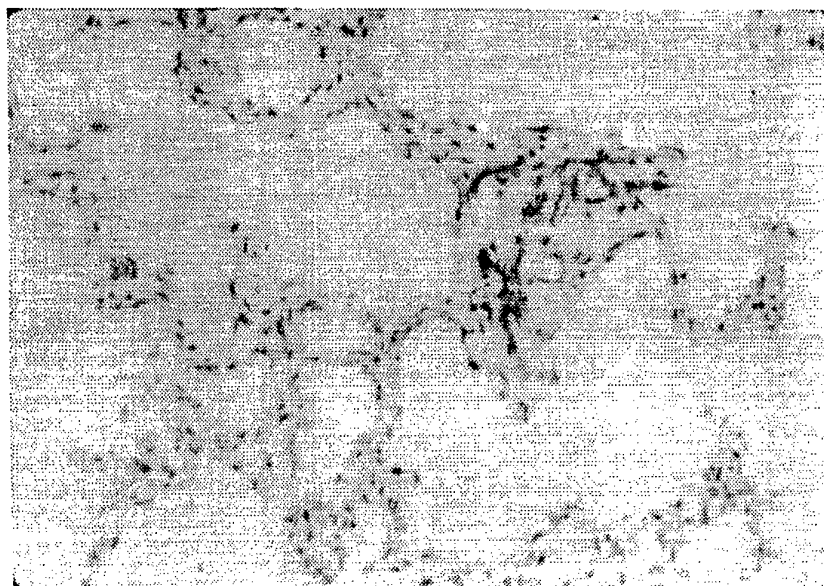


4/7

FIG. 1G



FIG. 1H



5/7

FIG. 1J

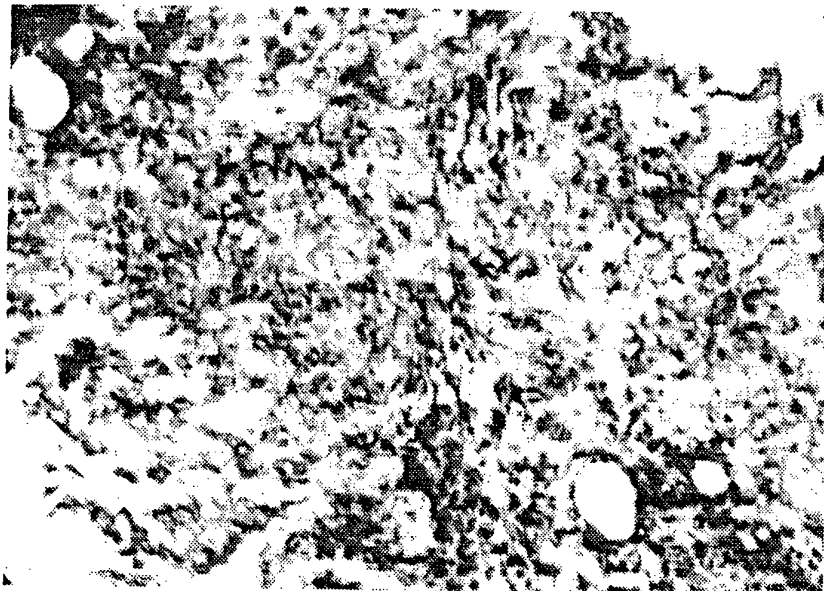
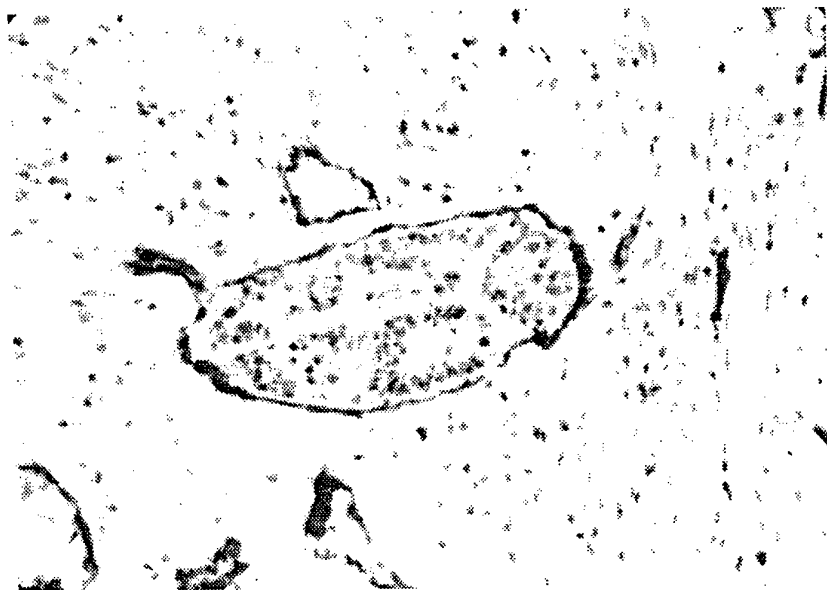


FIG. 1K



6/7

FIG. 1L

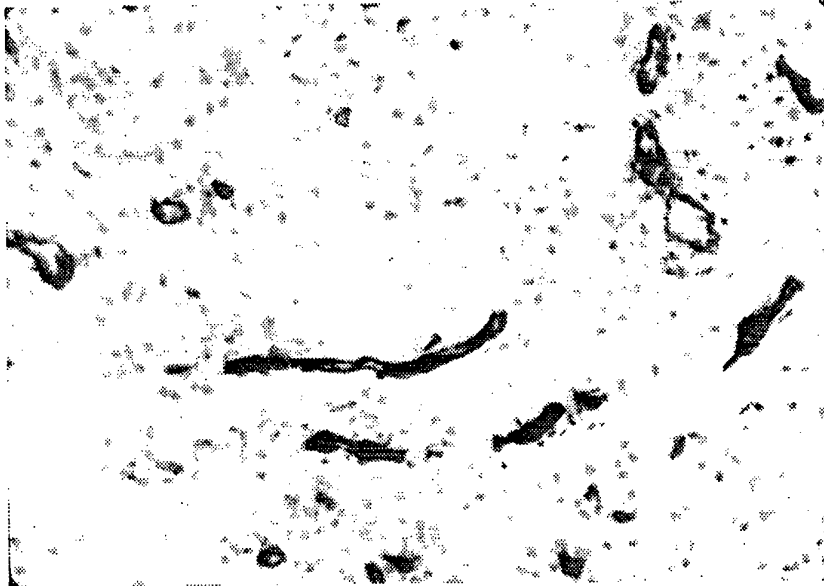
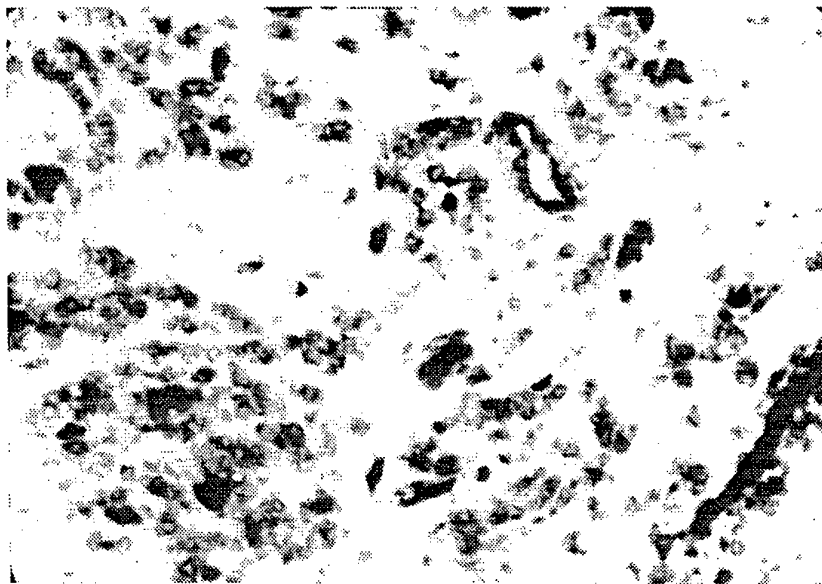


FIG. 1M



7/7

FIG. 2B

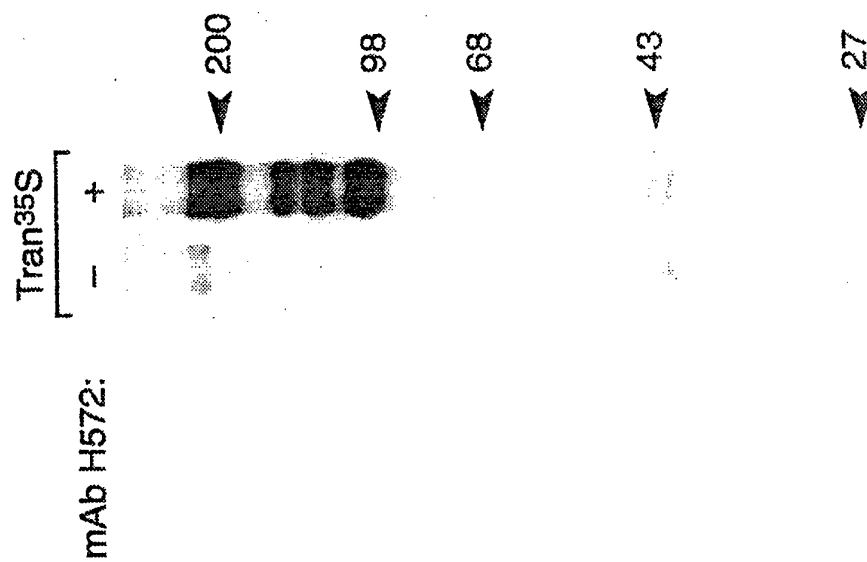
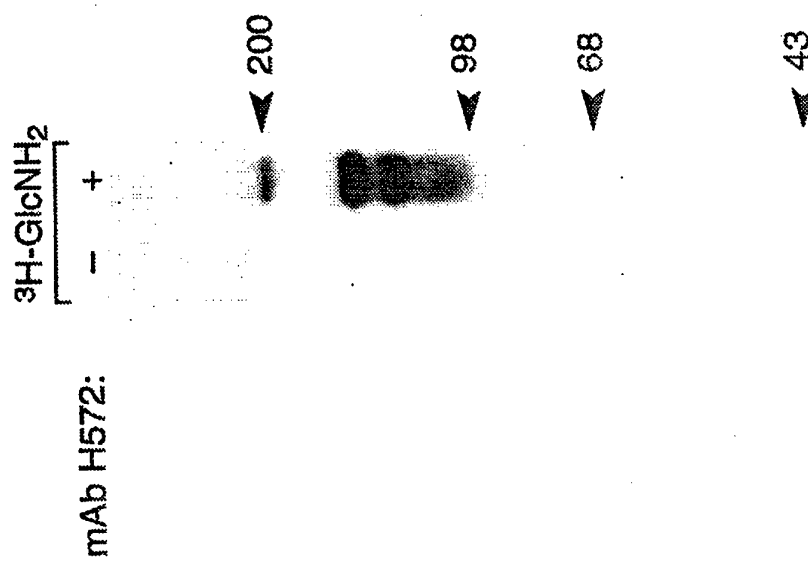


FIG. 2A



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05921

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 16/28, 2/00; C12N 5/20, 1/00; G01N 33/53; A61K 39/395

US CL : 530/387.3, 388.2, 350.; 425/240.27, 243, 7.21; 424/133.1, 141.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3, 388.2, 350.; 425/240.27, 243, 7.21; 424/133.1, 141.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL, VOLUME 62, ISSUED 13 JULY 1990, OSBORNE ET AL., "LEUKOCYTE ADHESION TO ENDOTHELIUM IN INFLAMMATION", PAGES 3-6, SEE ENTIRE DOCUMENT.	1-21
A	NATURE, VOLUME 346, NO. 6283, ISSUED 02 AUGUST 1990, SPRINGER ET AL., "ADHESION RECEPTORS OF THE IMMUNE SYSTEM", PAGES 425-434, SEE ENTIRE DOCUMENT.	1-21
A	US, A, 5,011,778 (NEWMAN ET AL.) 30 APRIL 1991, SEE ENTIRE DOCUMENT.	1-21
A	CLINICAL CHEMISTRY, VOLUME 27, NUMBER 11, ISSUED 1981, SEVIER ET AL., "MONOCLONAL ANTIBODIES IN CLINICAL IMMUNOLOGY", PAGES 1797-1806, SEE ENTIRE DOCUMENT.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

04 AUGUST 1995

Date of mailing of the international search report

17 AUG 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PAULA HUTZELL

Telephone No. (703) 308-0196