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TUMOR, CARBOHYDRATE ANTIGEN SPECIFIC MONOCLONAL ANTIBODY AND CELL LINE

The present invention relates to a novel
5 monoclonal tumour antigen specific antibody, a cell line
which produces the antibody, a pharmaceutical composition
comprising the monoclonal antibody, and the use of the
antibody in diagnosis and therapy, and the use of the cell
line for the production of the antibody.

10 The hybridoma technology for the production of
monoclonal antibodies, which was first described by Köhler
and Milstein (Nature, 256, 495-497, 1975), is nowadays well
established. By this technology myeloma cells are fused to
lymphocytes from animals which have been immunized with a
15 particular antigen. The resulting hybridoma cell produces
antibodies specific against a single antigenic determinant.
Monoclonal antibodies have therefore to a large extent
begun to replace conventional antisera in diagnostic
standard kits for immunoassays. Significant research has
20 also been done to adapt hybridoma technology for
therapeutic purposes.

It is further well-known that the
transformation of normal tissue cells to tumour cells is
associated with a change of the carbohydrate structure on
25 the cell surface. Many carbohydrate structures serve as
antigens, and the tumour-modified structures represent a
type of so-called tumour-associated antigens (abbreviated
TAA). The cell surface carbohydrate structures are linked
to either a lipid moiety, in which case they are called
30 glycolipids, or to proteins or peptides, in which case they
are called glycoproteins or glycopeptides, respectively. A
common designation for the two forms is glycoconjugate.

Tumour-associated glycoconjugate antigens are
previously known in relation to human tumour diseases.
35 Thus, two carcinoma associated antigens, CEA (carcinoma
embryonal antigen) and GICA (gastrointestinal cancer
antigen) carrying the epitope CA 19-9 have been
demonstrated particularly in gastrointestinal carcinomas,
while another third epitope, CA-50, seems to be a general

carcinoma antigen. All these antigens are secreted from the tumour cell surface and can be demonstrated in blood serum. These discoveries have urged the search for monoclonal antibodies which recognize tumour-specific antigens and which may be used in the immunolocalization and immunotherapy of various cancer diseases.

Monoclonal antibodies having pancreatic and colorectal cancer specificity are mentioned in several prior publications and conference abstracts under the designation C242 (corresponding antigen CA-242); see e.g. Larsson L.N. et al., *Int. J. Cancer* 42 (1988) 877-882, Lindholm L. et al., "An Immunoradiometric Assay (IRMA) for the CA-50 antigen", *Tumor Marker Antigens*, Ed. Jan Holmgren, Studentlitteratur 1985, Haglund C. et al., *Br. J. Cancer* 60 (1989) 845-51, Sell S., *Human Pathology* 21:10 (1990) 1003-19, Johansson C. et al., *Int. J. Cancer* 48 (1991) 757-763, and Kuusela P. et al., *Br. J. Cancer* 63 (1991) 636-40. Such C242 antibody has, however, never been disclosed previously more specifically or to such an extent that it could be produced by a person skilled in the art, and it has hitherto never been publicly available.

The present invention thus relates to a novel monoclonal antibody having significant specificity for gastrointestinal, in particular pancreatic and colorectal, cancer cells, a specific such monoclonal antibody being that which hereinafter is designated as C242:II.

C242:II is a monoclonal murine antibody of IgG class produced when culturing in an appropriate medium a hybridoma cell line obtained by fusing spleen cells from a mouse, which has been immunized with a human colonic adenocarcinoma cell line, with the murine myeloma cell line Sp2/0, as will be described in more detail in Example 1 below. A hybridoma cell line producing the C242:II antibody was deposited on January 26, 1990 in accordance with the Budapest Treaty at the European Collection of Animal Cell Cultures (ECACC), PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury Wilts., U.K., where it obtained the depository accession number 90012601.

Reference to this deposit has been made in our copending international patent application PCT/SE91/00496 (WO-A-9201470) that has priority from July 20, 1990.

5 In one aspect the present invention provides a novel monoclonal antibody which is the monoclonal antibody C242:II or an antibody having substantially the same properties, i.e. a functional equivalent, hereinafter commonly referred to as "antibody according to the invention".

10 In a further aspect the invention provides a cell line capable of producing the antibody according to the invention, particularly the above mentioned deposited hybridoma cell line 90012601.

15 By the expressions "substantially the same properties" and "functional equivalent" is meant that the antibody should have at least substantially the same immunologic specificity as that produced by the deposited cell line 90012601, i.e. bind to the same antigenic determinant or epitope, and compete with the C242:II
20 antibody for binding at that site.

Within the scope of the expression "antibody according to the invention" is also intended to be comprised antigen binding fragments of the antibody. Such fragments will retain the binding capability and
25 specificity of the unfragmented immunoglobulin and may, for example, be obtained by degrading the antibody by proteolytic enzymes, such as pepsin. The latter gives rise to a $F(ab')_2$ molecule and smaller peptides, the $F(ab')_2$ part being an example of such an antigen binding fragment.
30 Also comprised are corresponding antibodies or fragments produced by genetic engineering as well as derivatized or humanized forms of the antibody. Comprised within the inventive concept are antibodies deriving from mouse and other animals of different Ig classes/subclasses having
35 the same specificity. With respect to antibody-active fragments and recombinantly produced variants, the class/subclass of an antibody according to the invention may become less important because the unique class/subclass

specific determinants may be more or less omitted in the constructs. Thus the inventive concept spans from fully non-human to fully human antibodies and includes different chimeric forms. Fully human antibodies may be obtained by
5 culturing immortalized human antibody producing cells.

A certain aspect of the invention comprises antibodies that have a binding affinity/avidity for the CA-242 antigen/epitope which is up to 10^{+1} times, and in some cases down to 10^{-1} times, the binding affinity/avidity of the
10 antibody produced by the deposited hybridoma cell line. The crossreactivity in question is measured by blocking/binding assays as normally contemplated within the field. These affinity/avidity ranges apply in particular to unfragmented and underivatized non-human antibodies.

15 With respect to genetic engineering as mentioned above, the cDNA's of the variable regions of the light and heavy chains of the C242:II monoclonal antibody were cloned as will be described further in Example 4 below. Before discussing this in any more detail it may not be out of place
20 to give a brief general description of the basic immunoglobulin structural unit, reference being made to Fig. 1 of the accompanying drawings which describes the general structure of an antibody, i.e. immunoglobulin, of class G.

Referring to Fig. 1, the immunoglobulin consists
25 of two identical light (L) polypeptide chains and two identical heavy polypeptide chains (H), the four chains being joined by disulphide bonds and various non-covalent forces in a symmetric "Y"-configuration. Each heavy chain has a variable domain (V_H) at each end followed by a number of
30 constant domains (C_H), and each light chain has a variable domain (V_L) at one end and a constant domain (C_L) at the other end. There are two types of light chains, designated kappa and lambda, respectively. The variable domain of the light chain is aligned with the variable domain of the heavy
35 chain, and the constant domain of the light chain is aligned with the first constant domain of the heavy chain, the variable domains of the light and heavy chains forming the antigen binding site.

The variable domains of the light and heavy chains have the same general structure, each comprising three hypervariable regions, called complementarity determining regions, or CDR's, intervening between four framework regions, or FR's. The CDR's of each variable domain are kept in close proximity by the framework regions, and the two sets of CDR's together provide for the specificity of the antibody.

In order to clone the cDNA's of the variable portions of the light and heavy chains of the C242:II antibody, a cDNA phage library was first prepared from the C242:II hybridoma by per se known methods. Hybridization probes covering constant parts of the heavy chain and the kappa light chain, respectively, were then prepared from mouse genomic DNA using the polymerase chain reaction (PCR) and suitable primers. The resulting probes were used to screen the cDNA library for cDNA clones containing DNA sequences encoding the heavy and kappa chains of the C242:II antibody. Positively hybridizing phage clones were expanded and the cDNA was excised in the form of plasmids. The latter were characterized by restriction enzyme mapping, and plasmids containing inserts of the expected sizes were sequenced. In order to determine the CDR regions, the amino acid sequences encoded by the sequenced inserts were compared with those of previously known mouse kappa and heavy chains, considering the basic locations of CDR regions as defined by, e.g., Kabat E.A., et al. (1987), Sequences of Proteins of Immunological Interest, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health. It was found that the CDR's of the respective chains had the following amino acid sequences (as are also shown in Fig. 3 and 4 of the accompanying drawings):

Kappa chain/Light chain

CDR1:
ArgSerSerLysSerLeuLeuHisSerAsnGlyAsnThrTyrLeuTyr
(TrpPhe)

CDR2:

ArgMetSerAsnLeuValSer(GlyVal)

CDR3:

LeuGlnHisLeuGluTyrProPheThr(PheGly)

5 Heavy chain

CDR1:

(PheThr)TyrTyrGlyMetAsn

CDR2:

(MetGly)TrpIleAspThrThrThrGlyGluProThrTyrAlaGluAspPheLys

10 Gly(ArgIle)

CDR3:

(AlaArg)ArgGlyProTyrAsnTrpTyrPheAspVal(TrpGly)

The terminal dipeptide residues within brackets are optional variations of the CDR sequences. The kappa chain in figure 3 contains a cys residue meaning that the amino-terminal limits of its CDRs are well-defined.

The DNA sequences encoding the above CDR's are illustrated in Fig. 4. With the knowledge of these CDR amino acid and/or DNA sequences the skilled person may introduce the CDR regions of the C242:II antibody into the cloned variable parts of another antibody or antibody fragment by means of site-directed mutagenesis as is per se known in the art. Such procedure, generally known as "CDR grafting", involves substitution at DNA level of the CDR encoding sequences of the C242:II antibody for the CDR encoding sequences of the recipient antibody or fragment and will result in an antibody or antibody fragment having a corresponding specificity as the C242:II monoclonal antibody. Included within the scope of the antibody according to the invention is therefore, of course, any antibody or antibody fragment comprising the above defined CDR's or alternative CDR amino acid sequences that grafted onto functional antibody framework regions cross react with the antibody produced by the deposited hybridoma cell line ECACC 90012601.

The antibody according to the invention is directed against a tumour associated antigen, designated herein as CA-242, which appears to be a sialylated

carbohydrate antigen expressed on the same macromolecule as the antigen CA-50 mentioned above. Structurally, this antigen differs from the CA-50 epitope in that the antibody according to the invention does not react with either

5 sialylated Lewis-a substance or sialosyllacto-N-tetraose. A monoclonal antibody against CA-50 can, however, inhibit the binding of the antibody according to the invention to CA-50, suggesting a structural relation between CA-50 and the antigen recognized by the antibody according to the

10 invention. The tumour-associated antigen CA-242 is expressed in the vast majority of colo-rectal tumours and is only weakly expressed or absent in normal colonic tissue.

Following binding to cellbound antigen of the

15 antibody according to the invention, the antigen-antibody complex so formed is capable of being endocytosed or internalized into the cells. This is a prominent property of the C242:II antibody as deposited.

Although the antigen recognized by the antibody

20 according to the invention is very poorly expressed in normal pancreatic and colonic tissue, it is strongly expressed in pancreatic carcinoma and colonic adenocarcinoma cells. The antibody according to the invention would therefore find use in immunolocalization as

25 well as therapy of human pancreatic cancer and human colonic carcinoma.

In still another aspect the present invention thus provides the use of the novel antibody according to the invention in immunoassay or therapy.

30 In a further aspect the present invention provides a pharmaceutical composition comprising the novel antibody according to the invention.

An immunoassay for in vitro testing based upon the novel antibody according to the invention may be

35 designed in accordance with per se conventional immunological techniques in the art, utilizing the antibody according to the invention in a labelled or unlabelled form and determining the complex formation of the antibody with

specific antigenic species in the sample to be tested. In the first case the antibody may be labelled with a detectable label, such as a radiolabel, a chemiluminescer, a fluorescer or an enzyme label, whereas in the latter case the antibody is detected via a complex formed with a labelled substance or by non-labelling techniques, such as biosensor methods, e.g. based upon surface plasmon resonance. The sample may, for example, be in the form of a body fluid, such as serum, or a tissue preparation (histochemical assay).

For in vivo diagnostic purposes the antibody according to the invention is provided with a suitable externally detectable label, such as e.g. a radiolabel or a heavy metal atom, and administered to a subject whereupon the possible localized accumulation of antibody in the body is determined.

For therapeutical use the novel antibody according to the invention may be formulated in a variety of pharmaceutical compositions and preparations with pharmaceutically acceptable carriers, such as e.g. water, and be administered in various conventional ways, e.g. intravenously, subcutaneously, intramuscularly or intraperitoneally.

In the following non-limiting examples the preparation of a hybridoma producing the novel antibody according to the invention will be described, the use thereof in immunohistological evaluation, endocytosis thereof, and the cloning of cDNA's encoding the light and heavy chains of the C242:II monoclonal antibody. Reference will be made to the accompanying drawings wherein:

Fig. 1 is a schematic representation of the general immunoglobulin G structure;

Fig. 2 is a graph showing the endocytosis of C242:II cells;

Fig. 3 is a representation of the detailed cDNA sequence encoding the variable domain of the kappa chain of the C242:II monoclonal antibody along with the corresponding amino acid sequence; and

Fig. 4 is a representation of the detailed cDNA sequence encoding the variable domain of the heavy chain of the C242:II monoclonal antibody along with the corresponding amino acid sequence.

5

EXAMPLE 1

**Establishment of hybridoma cell line and production of
C242:II**

Preparation of established spleen cells

A human colorectal carcinoma cell line, COLO
10 205, commercially obtainable from the American Type Culture
Collection (ATCC), Rockville, Md., USA, under the accession
No. CCL 222, was routinely cultered in Iscove's MEM
complemented with 10% fetal calf serum (FCS). Cells were
harvested prior to confluence, normally 2 to 3 days after
15 subcultivation, and washed 3 times with phosphate buffered
saline (PBS) for immunization.

BALB/c mice, 4 to 6 weeks old, were immunized
intraperitoneally with a priming dose of 3×10^7 COLO 205
cells suspended in 0.1 ml of PBS. The animals were then
20 boosted with another 0.1 ml suspension of 3×10^7 COLO 205
cells and sacrificed four days later and the spleens were
removed. The spleens were then dissociated into a single
cell suspension.

Preparation of hybridoma

25 1.2×10^8 spleen cells from the above described
cell suspension were distributed among two tubes. To each
tube were additionally added 10^8 myeloma cells from the
mouse myeloma cell line Sp2/0 (available from the
collection of American Type Culture Collection (ATCC),
30 Rockville, Maryland, U.S.A. under the accession No. CRL
1581). The two tubes were centrifuged, and all liquid was
decanted. To each tube were then slowly added 2 ml of 37°C
PEG solution (10 g of PEG M_w 4000, 1 ml of DMSO and 10 ml
of monoclonal saline) over 1 minute and with constant
35 stirring. The tubes were transferred to a water-bath at
37°C for 90 seconds with gentle stirring. The fusion was
interrupted by adding to each test tube a physiological
buffer solution according to the following scheme: 2 ml

during the first 30 seconds, 6 ml during the following 30 seconds and another 32 ml over 1 minute. After washing of the cell suspension in culture medium it was suspended in totally 100 ml of hypoxanthine/aminopterin/thymidine (HAT) supplemented culture medium. The culture medium was
5 Iscove's MEM supplemented with 10% FCS, L-asparagine (36 mg/l), L-arginine-HCl (116 mg/l), folic acid (10 mg/l), L-glutamine (292.3 mg/l), sodium pyruvate (110.1 mg/l) and mercaptoethanol (3.49 μ l/l). Aliquotes of 50 μ l were
10 distributed to the wells of 96-well tissue culture dishes. The wells had been precoated with 250 μ l of macrophage suspension from BALB/c mice (2×10^4 cells/ml) in HAT supplemented culture medium. Medium was changed 6 days after fusion.

15

Screening of antibody secreting hybridomas

The spent medium from day 6 above was tested for COLO 205 positive antibodies as described by Kennett R.H., "Enzyme-linked antibody assay with cells attached to
20 polyvinyl chloride plates". Monoclonal Antibodies. Hybridomas. A new dimension in biological analyses. Eds. H.R. Kennett, T.J. McKevin, K.B. Bechtol, Plenum Press, New York 1980. In brief, the wells of Nunc immunoplates type IIF were coated with 2×10^5 cells/well of COLO 205 cells
25 (1 mg/100 ml PBS); coating volume 50 μ l. The above mentioned spent culture medium from day 6 was then added to the coated wells, 50 μ l/well. After incubation over night at room temperature peroxidase conjugated anti-mouse Ig (Dakopatts P260, Dakopatts A/S, Copenhagen, Denmark)
30 diluted (1/500) in 1% BSA-PBS was added at 100 μ g/well and incubated over night at room temperature. Substrate, in the form of 4 OPD-tablets Dako S2000 dissolved in 12 ml of citric acid buffer, pH 5.0, plus 5 μ l of 30% hydrogen peroxide, was then added at 100 μ l/well and the absorbance
35 was measured at 450 nm following incubation.

Roughly 600 hybridoma clones were tested. Initially, 190 of these reacted with COLO 205 cells. Of these, 177 reacted also with normal human lymphocytes

prepared on Lymphoprep from Nyegaard A/S, Norway, and were discarded. A clone established from one of the remaining clones was designated hybridoma C242 and was isotyped as IgG1 class. The C242 hybridoma was then subjected to a number of subcloning steps to eventually produce a final, stable monoclonal antibody producing a clone designated as C242:II.

Thus, the C242 hybridoma was first cloned resulting in a clone called C242:5. This clone was in turn cloned to form clone C242:5:2. In both these clonings, hybridoma suspension was diluted in hypoxanthine/thymidine (HT) supplemented culture medium to a density of 4 cells/ml. 50 μ l (0.2 cells) were distributed to each well in a 96-well culture dish pre-coated with 250 μ l of Balb/c mice macrophage suspension (5×10^3 macrophages/well) in HT supplemented medium. After 2-5 days, single cell clones were detected by visual inspection in a microscope. On day 6, medium was changed and aliquots of spent media analyzed for quantity of antibodies binding to COLO 205 cells but not to normal human cells by ELISA as described above. The best clones in terms of positive reaction in the COLO 205 ELISA with retained negative reaction in the normal cell ELISA were selected and frozen in vials in liquid nitrogen for further development.

For performing a third cloning, the cells were thawed and cultured in DMEM (Dulbecco's Modified Eagle's Medium) (5% FCS). Cells were then seeded into the wells of a 96-well tissue culture plate at a mean density of three cells per well. The cloning was performed in DMEM with 5% FCS and mouse macrophages used as feeder cells. On this plate, 30 clones occurred of which 16 were tested for IgG production by nephelometry and specificity of the antibodies by ELISA as described above. Twelve of these clones were found to produce IgG. Ten clones were then expanded on a 24-well tissue culture plate from which spent medium was tested for IgG production and specificity. This selection resulted in the saving of 4 clones with relatively high productivity. A final productivity test of

these four clones was performed in duplicate tissue culture flasks. The clone showing the highest productivity was selected and designated as C242/CL 510 A1. It was frozen in liquid nitrogen for further use.

5 A first cloning of the clone C242/CL 510 A1 indicated that one third of the cells did not produce IgG as judged by an absorbance of less than 0.1 at a dilution of 1:1000 in a general mouse IgG ELISA. Five positive subclones were pooled to create a clone called C242:I. The
10 productivity from this clone was quantified by a general HPLC-based assay to be 150 μ g of mouse IgG per ml.

Clone C242:I was subsequently cloned into a 96-well dish yielding 33 clones, all positive for mouse IgG production. Five of these, all yielding above 150 μ g/ml,
15 were pooled to create a final clone, designated as C242:II, having a stable productivity of IgG. HPLC assay indicated the productivity of C242:II to be 196 μ g of mouse IgG per ml. The cells were frozen and stored in vials in liquid
20 nitrogen. A sample of the C242:II hybridoma produced was deposited at the ECACC under accession number 90012601 as stated above.

Production of C242:II monoclonal antibody

An initial cell suspension of the above prepared hybridoma was obtained from a frozen vial. Cells were seeded into tissue culture chambers with a total area of 6000 cm² from Nunc A/S at a density of 2×10^4 cells/ml (C242:II hybridoma). The cells were then cultured in DMEM modified according to Iscove (Iscove N. N., and Melchers F., J. Exp. Med. vol. 147, p 923 (1978) and supplemented with 1% gentamycin, 1% amino-acid supplement and 5% fetal calf serum. The cells were then incubated for 4 or 5 days at 37°C in a humidified atmosphere containing 8% CO₂. At the end of incubation cells were counted and samples were taken for determination of monoclonal antibody concentration. The cell culture supernatant was decanted and filtered through a cellulose filter in order to remove suspended cells. The supernatant was then concentrated 20-30 times by ultrafiltration in a Millipore Pellican Ultrafiltration cell using a 30,000 molecular weight cut-off membrane. A sample from the concentrate was taken for analysis of C242:II monoclonal antibody concentration and antigen reactivity, whereupon the monoclonal antibody concentrate was stored at -70°C until purification.

Purification of C242:II monoclonal antibody

Protein-A-Sepharose^R 4B (Pharmacia AB, Uppsala, Sweden) was prepared according to the manufacturer's instructions concerning swelling and washing of the gel, whereupon the above produced C242:II antibody concentrate was bound thereto using 1.5 M glycine-NaOH, 3 M NaCl, pH 8.9, as binding buffer. After an initial washing using the same buffer, the affinity column was eluted with 0.1 M citric acid-NaOH, pH 5.0, and the C242:II monoclonal antibody was collected in tubes containing 1 mole/l of Tris-HCl, pH 8.0. The antibody obtained was then dialyzed against 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2, 0.2 g/l NaN₃. The dialyzed C242:II antibody was then concentrated by ultrafiltration using a 30,000 molecular weight cut-off membrane. After taking of samples for

concentration and quality control analysis the C242:II monoclonal was stored at -20°C .

EXAMPLE 2

5 **Immunohistochemical evaluation of C242:II in colorectal cancer and normal tissues**

Specimens from primary colo-rectal carcinoma and various normal tissues were obtained from patients undergoing surgical resection. The tissues were kept on ice and were frozen within 1 hour after resection in iso-pentane pre-chilled with liquid nitrogen. The tissues were stored at -70°C until sectioned. The frozen biopsies were cut into $5\ \mu\text{m}$ sections and fixed in 50% acetone for 30 seconds at $+4^{\circ}\text{C}$ followed by 100% acetone for 5 minutes at $+4^{\circ}\text{C}$. The sections were air-dried and rinsed in PBS for 10 minutes. All sections were then incubated in 0.3% hydrogen peroxide in PBS for 5 minutes to block endogenous peroxidase and rinsed twice in PBS. Thereafter the sections were treated with normal swine serum and diluted 1:10 in PBS-4% BSA for 5 minutes at $+4^{\circ}\text{C}$ to block non-specific binding of antibodies.

All incubations with antibodies were carried out in a humid atmosphere for 30 minutes at room temperature. The sections were first incubated with monoclonal antibody C242:II, obtained in Example 1 above, in PBS-4% BSA, thereafter with biotinylated horse anti-mouse IgG (VectastainTM, Vector Laboratories, Burlingame, CA, U.S.A.) diluted 1:400 in PBS-4% BSA with 2% swine anti-rabbit immunoglobulin and finally with Avidin DH/biotinylated horseradish peroxidase H complex (Dakopatts A/S, Copenhagen, Denmark). After each incubation the slides were rinsed in PBS for 15 minutes. The sections were then treated with substrate for 15 minutes, rinsed in PBS, counterstained with haematoxylin and mounted with glycerol-gelatin (Merck, Darmstadt, Germany). The substrate used was 10 mg of 3-amino-9-ethylcarbazole (Sigma, St. Louis, Mo., USA) dissolved in 6 ml of dimethylsulfoxide and diluted with 50 ml of 0.02 M sodium acetate, pH 5.5, containing 4

μ l of 30% H₂O₂. The substrate solution was filtered prior to use. The sections were then examined and the results are presented in Table 1 below.

TABLE 1

5

Staining of colo-rectal tumours and various normal tissues with C242:II	
<u>TISSUE</u>	<u>STAINED/TOTAL</u>
Colo-rectal carcinoma	26/41
10 normal colon	8/16
mammary gland	2/3
parotid gland	2/2
skin	0/1 slight staining of sweat glands
15 liver	0/2 weak staining of bile ducts
kidney	0/1
pancreas	2/2 ducts
stomach	0/1
20 small intestine	0/1

As appears from Table 1, 63% of the examined biopsies from colo-rectal tumours showed positive staining with monoclonal C242:II. The expression of the antigen CA242 in normal colonic tissue was found to be generally weaker than in tumour tissue, the staining being entirely localized to columnar epithelium and goblet cells. Non-colon normal tissue was almost devoid of reactivity with monoclonal C242:II, whereas positivity was found in normal breast ducts, pancreatic ducts, bile ducts, and sweat glands, but the intensity of staining was weak.

25
30

EXAMPLE 3**Endocytosis of C242:II binding to COLO 205 human colon carcinoma**

35

An endocytosis assay was conducted as follows:

Single cell suspensions were prepared from COLO 205 cells (see Example 1) grown as a monolayer culture. The cells were trypsinized, extensively washed and resuspended in culture medium. Iodine labelled C242:II antibody from Example 1 (200 000 cpm corresponding to 50 ng of monoclonal antibody) was added to a single cell suspension of COLO 205 cells (10^6 cells) in 5 ml test tubes. The final volume was 200 μ l/tube.

Cells were incubated in the presence of ^{125}I -C-242 for 1 hour on ice (0°C). The suspension was then centrifuged and the cells were washed free from unbound monoclonal antibody. The preincubated cells were further incubated at 37°C for 10 minutes time intervals during 1 hour, the cells being rechilled and pelleted at each time. Radiolabel released to the culture medium was measured from the supernatant (LKB gamma counter, Pharmacia AB, Uppsala, Sweden). The supernatant was also subjected to TCA precipitation and the radioactivity of the precipitate was measured. Surface bound ^{125}I -C242 was removed by acid wash with 0.2 M glycine-HCl buffer, pH 1.5, containing 2.5 mg/ml papain, and the radioactivity, representing internalized ^{125}I -C242, was counted. The amount of surface bound antibody after each time, incubated at 37°C , was calculated by subtracting released and internalized monoclonal antibody from total surface bound monoclonal antibody. Degradation of released antibody was measured from TCA precipitation of medium supernatant.

The results are presented in Fig. 1 in the accompanying drawing showing the endocytosis of ^{125}I -C242:II by COLO 205 cells, the radioactivity on cell surface ("Sur"), internalized radioactivity ("Int"), released radioactivity ("Res") and degraded released radioactivity ("Deg.Re") being expressed as the percentage of total initial radioactivity on cell surface. In Fig. 1 it can be seen that a more than 20% internalization of C242:II was obtained within 1 hour when preincubated cells were incubated at 37°C . There were small amounts of acid soluble radioactivity when medium supernatant was TCA

precipitated which indicates little fragmentation of released activity after 1 hour of incubation.

EXAMPLE 4

5 Molecular cloning of cDNA's encoding parts of the light and heavy chains of the monoclonal antibody C242:II

A. Preparation of total RNA

Total RNA from 10^8 cells was prepared essentially according to the method of Chirgwin, J.M. et al. (1979; Biochemistry 18, 5294-5299) as modified by
10 Chomezynski, P. and Sacchi, N, (1987; Anal. Biochem. 162, 156-159). Briefly, the cell pellet was dissolved in 15 ml of cold denaturing solution consisting of 4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.5% N-lauroyl
15 sarcosine; 0.1 M 2 mercaptoethanol. After the dissolution of the cell material 1.2 ml of 2 M sodium acetate (pH 4.0) was added and the mixture extracted with phenol-chloroform-isoamyl alcohol (250:49:1). After centrifugation the RNA was precipitated from the aqueous phase by adding an equal
20 volume of isopropanol, and incubating at -20°C over night in order to precipitate the total RNA. After centrifugation and resuspension of the RNA, the precipitation was repeated, and after an additional centrifugation and precipitation the total RNA yield was calculated to be 960
25 μg based on absorbance measurements.

B. Isolation of mRNA

In order to isolate polyadenylated mRNA from the above preparation of total RNA, the PolyAtractTM mRNA isolation system of Promega Corporation, Madison,
30 Wisconsin, U.S.A., was utilized according to the manufacturer's instructions (c.f.: Promega Technical Bullentin, No. 090: 1990). Briefly, 960 μg of total RNA were dissolved in water and annealed with a biotinylated oligo(dT) probe (50 pmoles) in 0.4 x SSC (1 x SSC= 8.77 g
35 NaCl, 4.41 g sodium citrate per liter of water at pH 7.0). Streptavidin coated paramagnetic beads (Streptavidin MagnesphereTM) were added and after a 10 min. incubation the magnetic particles were removed and washed several

times with 0.1 x SSC. The polyadenylated mRNA was eluted from the spheres by incubation in water, yielding 5 µg of mRNA.

C. Preparation of cDNA phage library in E. coli

5 cDNA was prepared from polyadenylated mRNA of the previous step essentially according to the method of Gubler, U., and Hoffman, B.J. (1983; Gene 25, 263-269), as modified for the vector Uni-ZAP™ (Stratagene Inc. La Jolla, California) which permits unidirectional cloning of
10 double stranded cDNA. Briefly, 5 µg of polyadenylated mRNA was converted to single stranded cDNA by priming with the Uni-ZAP™ linker primer (56 ng/ml), adding dATP, dGTP, dTTP, and 5-methyl-dCTP, at 0.6 mM, and 45 U of Moloncy Murine Leukemia Virus reverse transcriptase. The mixture
15 was incubated at 37 °C for 1 hours. Second strand cDNA synthesis was performed by adding dATP, dGTP, dTTP, and dCTP to a final concentration of 0.15 mM, 3.2 U RNase H, and 6.5 U DNA Polymerase 1, and incubating for 2.5 hours at
20 16 °C. The mixture was extracted with phenol-chloroform (1:1) and ethanol precipitated. The ends of the cDNA were rendered blunt by incubating with 0.16 mM dNTP and 10 U T4 DNA polymerase at 37 °C for 30 min. After phenol-chloroform extraction and ethanol precipitation, the resulting blunt-ended cDNA was ligated to EcoRI adaptors by adding 3 Weiss
25 U T4 DNA ligase, 1 mM ATP, and incubating over night at 8 °C. After ligation the EcoRI cohesive ends were kinased by adding 10 U T4 Polynucleotide kinase in the presence of 1 mM ATP, and incubating for 30 minutes at 37 °C. The resulting cDNA with phosphorylated EcoRI cohesive ends, was
30 digested with 90 U XhoI in order to create an XhoI cohesive end from the sequence encoded by the Uni-ZAP™ linker primer. The resulting cDNA was then ligated to 1 µg of Uni-ZAP™ XR EcoRI and XhoI prepared arms in the presence of 2 Weiss U T4 DNA ligase, 1 mM ATP, and incubating at 12 °C
35 overnight. The ligation mixture was packed in vitro into - phage particles by using the Gigapack II Gold^R packaging extract according to the manufacturer's instructions, and the resulting phages were used to infect E. coli PLK-F'.

the resulting cDNA library of 8.5×10^4 independent clones was amplified once to give a phage titer of 7.5×10^8 pfu/ml. The resulting cDNA library was screened using *E. coli* XL1-Blue as host strain (c.f. Bullock. W. (1978) Biotechniques 5, 4) as will be described below.

D. Preparation of hybridization probes

In order to detect cDNA clones encoding the IgG1 heavy chain and kappa chain of the C242:II antibody, hybridization probes covering the first constant domain of the heavy chain, CH1, and the constant domain of the kappa chain, CK, were prepared from mouse genomic DNA by the polymerase chain reaction according to Saiki. R.H., et al. (1988; Science 239, 487-491). Briefly, oligonucleotide primer pairs hybridizing to the 5' and 3' regions of the exons encoding the above immunoglobulin domains were used for amplification of mouse genomic DNA. After purification by agarose gel electrophoresis the resulting DNA probe fragments were labeled with ^{32}P by the random primer extension method according to Feinberg, A.P., and Vogelstein, B. (1983; Anal. Biochem. 132, 6).

E. Screening for IgG1 heavy and kappa chains encoding sequences and insertion into plasmids

The cDNA library from section C above was screened in two separate rounds using the immunoglobulin IgG1 and kappa probes, respectively, from section D according to methods described by Sambrook, J. et al. (1989; Molecular Cloning, 2nd Ed, Cold Spring Harbor Laboratory Press). Positively hybridizing phage clones from the two separate hybridisations were further purified by rescreening using the same probes as in the initial screening. Positively hybridizing phage clones were expanded in cultures of *E. coli* XL1-Blue, and the resulting phage stocks used to prepare cDNA containing pBluescript SK(-) plasmids by phagemid excision and propagation essentially according to Short, J.M. et al. (1988; Nucleic Acids Res. 16, 7583-7600).

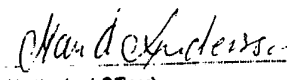
F. Characterization of plasmid cDNA from hybridizing colonies

The resulting cDNA containing plasmids were characterised by restriction enzyme mapping, and plasmids containing inserts of the expected sizes were subjected to dideoxy DNA sequencing according to Sanger, F. et al. (1977; Proc. Natl. Acad. Sci. USA 74, 5463-5467). A plasmid hybridizing with the Ig kappa probe, denoted pKGE761, contained a cDNA insert whose sequence encodes an open reading frame closely homologous to previously known mouse kappa light chain sequences (c.f. Kabat, E.A. et al. (1987) Sequences of Protein of Immunological Interest, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health). A partial cDNA sequence encoding the variable region of the kappa light chain, VK, and its translated protein sequence, is depicted in Fig. 3. The three CDR sequences, designated as CDR1 to CDR3, are indicated by underlining. A signal peptide preceding the aminoterminal end of the VK segment is indicated by S. CK indicates the constant region following the carboxyterminal end of the VK sequence.

A plasmid hybridizing with the IgG1 probe, denoted pKGE762, contained a cDNA insert whose sequence encodes an open reading frame closely homologous to previously known mouse IgG1 heavy chain sequences (c.f. Kabat, E.A., et al. vide supra). A partial cDNA sequence encoding the IgG1 heavy chain variable region, VH, and its translated protein sequence, is depicted in Fig. 4. The three CDR sequences, designated as CDR1 to CDR3, are indicated by underlining. A signal peptide preceding the aminoterminal end of the VH segment is indicated by S. CH1 indicates the constant region following the carboxyterminal end of the VH sequence.

20/1

International Application No: PCT/

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>2</u> , line <u>33-38</u> of the description ¹	
A. IDENTIFICATION OF DEPOSIT ²	
Further deposits are identified on an additional sheet <input type="checkbox"/> ³	
Name of depositary institution ⁴ European Collection of Animal Cell Cultures (ECACC)	
Address of depositary institution (including postal code and country) ⁵ PHLS Centre for Applied Microbiology and Research Porton Down, Salisbury Wilts., U.K.	
Date of deposit ⁶ January 26, 1990	Accession Number ⁶ 90012601
B. ADDITIONAL INDICATIONS ⁷ (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ⁸ (if the indications are not for all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS ⁹ (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later ⁹ (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
 (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau ¹⁰	
was	_____ (Authorized Officer)

CLAIMS

1. An antibody produced by hybridoma cell line
5 C242:II with ECACC identification number 90012601, or an
antibody having substantially the same antigen binding
properties (epitope specificity).
2. A monoclonal antibody according to claim 1 for
10 in vivo diagnostic use.
3. A monoclonal antibody according to claim 1 for
therapeutical use.
- 15 4. An antibody according to any of claims 1-3 in
fragmented form.
5. An antibody according to any of claims 1-4 which
binds specifically to the same epitope as an antibody that
20 in its CDRs have
Kappa chain:
CDR1: ArgSerSerLysSerLeuLeuHisSerAsnGlyAsnThrTyrLeuTyr
CDR2: ArgMetSerAsnLeuValSer
CDR3: LeuGlnHisLeuGluTyrProPheThr
25 Heavy chain:
CDR1: TyrTyrGlyMetAsn
CDR2: TrpIleAspThrThrThrGlyGluProThrTyrAlaGluAspPheLysGly
CDR3: ArgGlyProTyrAsnTrpTyrPheAspVal.
- 30 6. A method for in vitro diagnostics, comprising
the steps of contacting a sample with a monoclonal antibody
according to any of claims 1-5 under conditions permitting
formation of a complex containing the monoclonal antibody
bound to its antigen and subsequently determining the
35 complex formed, the amount of which is correlated to the
amount of antigen present in the sample.

7. A method for in vivo diagnostics, comprising the steps of administering a monoclonal antibody according to any of claims 1-5 to a subject and determining and localizing the accumulation thereof in the subject, the accumulation of which indicating the localization of a tumour expressing CA-242 antigen in said subject.
8. A method of treating tumours in a human or an animal, comprising administering an antibody according to any of claims 1-5.
9. A pharmaceutical composition, comprising a monoclonal antibody according to any of claims 1-5.
10. A pharmaceutical composition according to claim 7, comprising an aqueous solution of the monoclonal antibody.
11. A cell line capable of producing the antibody of or an antigen binding fragment thereof according to any of claims 1-5.
12. A cell line according to claim 11, which is hybridoma cell line C242:II with ECACC identification number 900012601, or an artificial or spontaneous variant thereof.
13. A monoclonal antibody which binds specifically to the same epitope as an antibody that in its CDRs have
- Kappa chain:
- CDR1: ArgSerSerLysSerLeuLeuHisSerAsnGlyAsnThrTyrLeuTyr
CDR2: ArgMetSerAsnLeuValSer
CDR3: LeuGlnHisLeuGluTyrProPheThr
- Heavy chain:
- CDR1: TyrTyrGlyMetAsn
CDR2: TrpIleAspThrThrThrGlyGluProThrTyrAlaGluAspPheLysGly
CDR3: ArgGlyProTyrAsnTrpTyrPheAspVal.

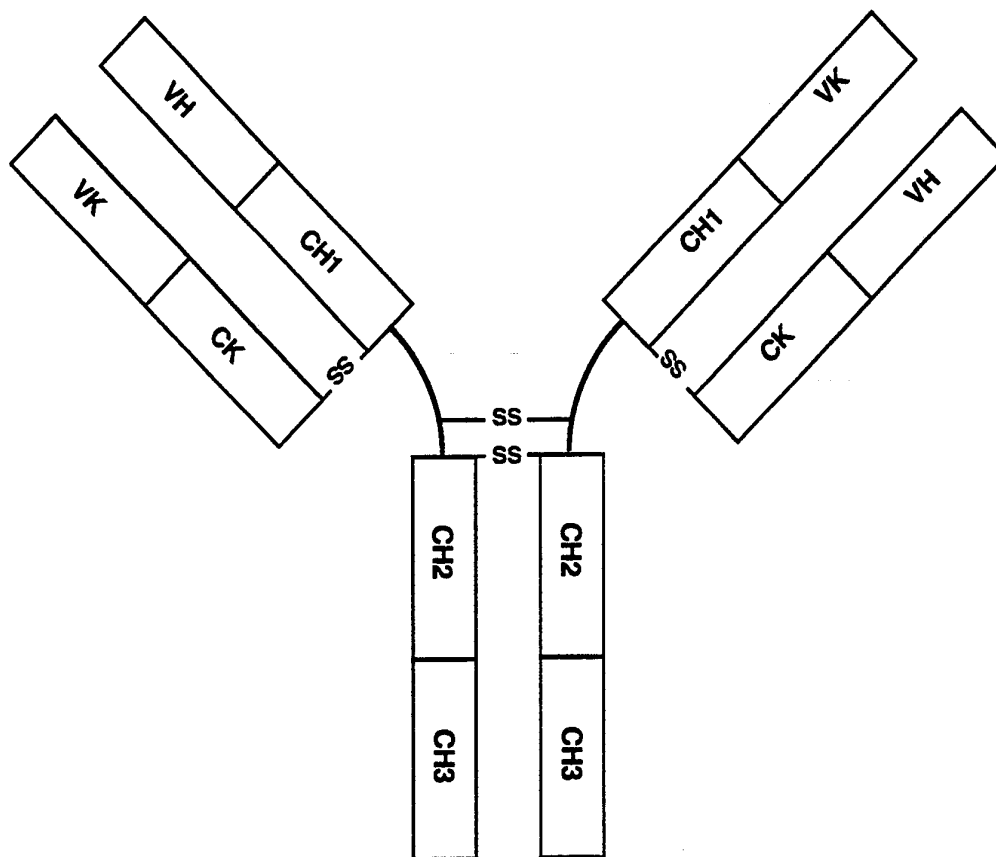


Fig. 1

Endocytosis; 125-I-C242 / Colo-205

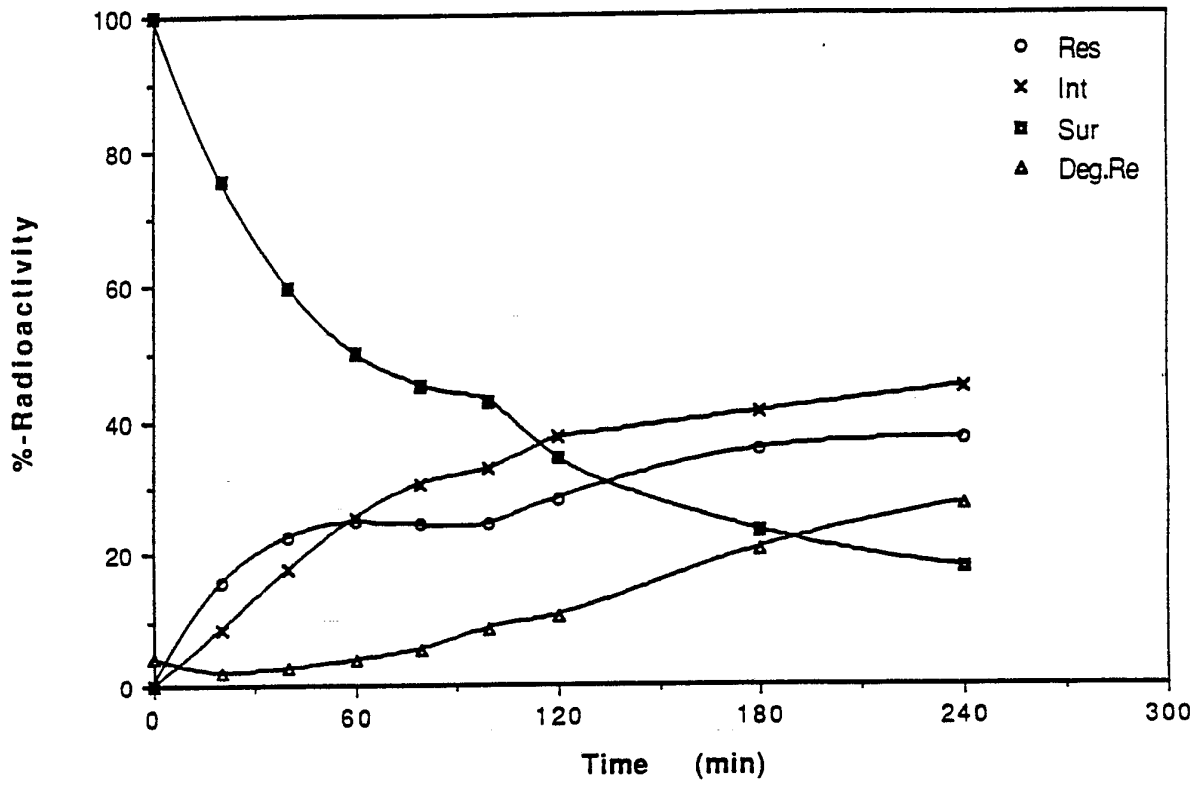


FIG.2

Fig. 3

1 GGAATTCGGCACGAGGAGTTTTTTTGTATCAAGTTCTCAGAATGAGGTGCCTAGCTGAGT 60
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 MetArgCysLeuAlaGluPhe -

61 TCCTGGGGCTGCTTGTGCTCTGGATCCCTGGAGCCATTGGGGATATTGTGATGACTCAGG 120
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 LeuGlyLeuLeuValLeuTrpIleProGlyAlaIleGlyAspIleValMetThrGlnAla -
 ↓VK →

121 CTGCACCCTCTGTACCTGTCACTCCTGGAGAGTCAGTATCCATCTCCTGCAGGTCTAGTA 180
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 AlaProSerValProValThrProGlyGluSerValSerIleSerCysArgSerSerLys -
 CDR1

181 AGAGTCTCCTGCATAGTAATGGCAACACTTACTTGTATTGGTTCCTGCAGAGGCCAGGCC 240
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 SerLeuLeuHisSerAsnGlyAsnThrTyrLeuTyrTrpPheLeuGlnArgProGlyGln -
 CDR1

241 AGTCTCCTCAGCTCCTGATATATCGGATGTCCAACCTTGTCTCAGGAGTCCCAGACAGGT 300
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 SerProGlnLeuLeuIleTyrArgMetSerAsnLeuValSerGlyValProAspArgPhe -
 CDR2

301 TCAGTGGCAGTGGGTGAGGAACTGCTTTACACTGAGAATCAGTAGAGTGGAGGCTGAGG 360
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 SerGlySerGlySerGlyThrAlaPheThrLeuArgIleSerArgValGluAlaGluAsp -

361 ATGTGGGTGTTTATTACTGTCTGCAACATCTAGAGTATCCGTTACGTTCCGGTCTGGGA 420
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 ValGlyValTyrTyrCysLeuGlnHisLeuGluTyrProPheThrPheGlyProGlyThr -
 CDR3

421 CCAAGCTGGAGCTGAAACGGGCTGATGCTGCACCAACTGTAACG 464
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 LysLeuGluLeuLysArgAlaAspAlaAlaProThrValThr -
 ← VK ↓CK →

Fig. 4

1 GAATTCGGCACGAGATTGAGCCCAAGTCTTAGACATCATGGATTGGCTGCGGAACCTTGCT
 -----+-----+-----+-----+-----+-----+-----+-----+
 MetAspTrpLeuArgAsnLeuLeu

61 ATTCCTGATGGCAGCTGCCCAAAGTATCCAAGCACAGGTCCAGTTGGTGCAGTCTGGACC
 -----+-----+-----+-----+-----+-----+-----+-----+
 PheLeuMetAlaAlaAlaGlnSerIleGlnAlaGlnValGlnLeuValGlnSerGlyPro
 ↓ VH →

121 TGAGCTGAAGAAGCCTGGAGAGACAGTCAAGATCTCCTGCAAGGCTTCTGATTATACCTT
 -----+-----+-----+-----+-----+-----+-----+-----+
 GluLeuLysLysProGlyGluThrValLysIleSerCysLysAlaSerAspTyrThrPhe

181 CACATACTATGGAATGAACTGGGTGAAGCAGGCTCCGGGAAAGGGTTTAAAGTGGATGGG
 -----+-----+-----+-----+-----+-----+-----+-----+
 ThrTyrTyrGlyMetAsnTrpValLysGlnAlaProGlyLysGlyLeuLysTrpMetGly
 CDR1

241 CTGGATAGACACCACCACTGGAGAGCCAACATATGCTGAAGATTTTAAAGGACGGATTGC
 -----+-----+-----+-----+-----+-----+-----+-----+
 TrpIleAspThrThrThrGlyGluProThrTyrAlaGluAspPheLysGlyArgIleAla
 CDR2

301 CTCTCTTTGGAGACCTCTGCCAGCACTGCCTATTTGCAGATCAAAAACCTCAAAAATGA
 -----+-----+-----+-----+-----+-----+-----+-----+
 PheSerLeuGluThrSerAlaSerThrAlaTyrLeuGlnIleLysAsnLeuLysAsnGlu

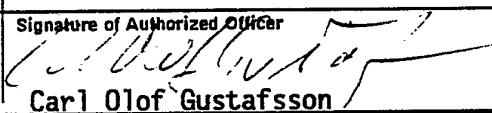
361 GGACACGGCTACATATTTCTGTGCAAGACGGGGCCTTACAACCTGGTACTTTGATGTCTG
 -----+-----+-----+-----+-----+-----+-----+-----+
 AspThrAlaThrTyrPheCysAlaArgArgGlyProTyrAsnTrpTyrPheAspValTrp
 CDR3

421 GGGCGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACGACNCCCCcaTCTGTCTATCC
 -----+-----+-----+-----+-----+-----+-----+-----+
 GlyAlaGlyThrThrValThrValSerSerAlaLysThrThrProProSerValTyrPro
 ← VH ↓ CH1 →

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00495

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: C 12 P 21/08, A 61 K 39/395, G 01 N 33/574		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	A 61 K; G 01 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	Br. J. Cancer, Vol. 63, 1991 P. Kuusela et al.: "Comparison of a new tumour marker CA 242 with CA 19-9, CA 50 and carcinoembryonic antigen (CEA) in digestive tract diseases", see page 636 - page 640 "Discussion" --	1-13
A	Br. J. Cancer, Vol. 60, 1989 C. Haglund et al.: "Tissue expression of the tumor associated antigen CA242 in benign and malignant pancreatic lesions. A comparison with CA 50 and CA 19-9", see page 845 - page 851 --	1-13
A	Int. J. Cancer, Vol. 42, 1988 L. N. Larson et al.: "Mouse monoclonal antibodies for experimental immunotherapy promotes killing of tumor cells", see page 877 - page 882 --	1-3,6,9-11
<p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"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</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"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.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
14th October 1992	16 -10- 1992	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	 Carl Olof Gustafsson	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Int. J. Cancer, Vol. 48, 1991 C. Johansson et al.: "Comparison of serological expression of different epitopes on the CA50-Carrying antigen CanAg", see page 757 - page 763 --	1,4
A	Cancer, Vol. 61, 1988 S. Itai et al.: "Significance of 2-3 and 2-6 Sialylation of Lewis A Antigen in Pancreas cancer", see page 785 - page 787 table 1 and page 785-7 --	1-3,6,9-11
T	The Journal of Biological Chemistry, Vol. 266, No. 23, 1991 E. L. Berg et al.: "A Carbohydrate Domain Common to Both Sialyl Le and Sialyl Le Is Recognized by the Endothelial Cell Leukocyte Adhesion Molecule ELAM-1", see page 14869 - page 14872, page 14870, right column - page 14871, left column --	1
X	US, A, 4752569 (P. I. TERASAKI ET AL.) 21 June 1988, see column 1-3 and table 4 --	1-3,6,9-11
X	Human Pathol., Vol. 21, No. 10, 1990 S. Sell: "Cancer-Associated Carbohydrates Identified by Monoclonal Antibodies", see page 1003 - page 1018 --	1-3,6,9-11
X.	US, A, 4904596 (S-I HAKOMORI ET AL.) 27 February 1990, see col. 2, lines 8-24, example 4 and col. 15-19 --	1-3,6,9-11
A	J. Natl. Cancer Inst., Vol. 78, No. 3, March 1987 M. Yuan et al.: "Expression of Lewis and Sialylated Lewis Antigens in Human Colorectal Polyps", see page 479 page 480, right column --	1-3,6,9-11
A	Cancer Research, Vol. 45, January 1985 D. Chia et al.: "Use of Monoclonal Antibodies to Sialylated Lewis and Sialylated Lewis for Serological Tests of Cancer", see page 435 - page 437 --	1

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Science, Vol. 250, 1990 G. Walz et al.: "Recognition by ELAM-1 of the Sialyl-Le Determinant on Myeloid and Tumor Cells", see page 1132 - page 1135 -- -----	1

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 7 and 8 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 92/00495

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/09/92. The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4752569	88-06-21	EP-A- 0165811 JP-A- 61063700	85-12-27 86-04-01
US-A- 4904596	90-02-27	JP-A- 62190074	87-08-20