(51) International Patent Classification 4:
C12N 5/00, C12P 21/00
C07K 15/00, C12N 15/00
G01N 33/574, 33/577, A61K 49/02
A61K 39/395, 43/00 // (C12P 21/00
C12R 1:91)

(11) International Publication Number:
WO 86/02945

(43) International Publication Date:
22 May 1986 (22.05.86)

(21) International Application Number:
PCT/US85/01511

(22) International Filing Date:
8 August 1985 (08.08.85)

(31) Priority Application Numbers:
670,328
702,059

(32) Priority Dates:
9 November 1984 (09.11.84)
15 February 1985 (15.02.85)

(33) Priority Country:
US

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(81) Designated States: AT (European patent), AU, BE (Eu-
ropean patent), BR, CH (European patent), DE (Eu-
ropean patent), DK, FR (European patent), GB (Eu-
ropean patent), HU, IT (European patent), JP, KR,
LU (European patent), NL (European patent), NO,
SE (European patent), SU.

Published
With international search report.

(54) Title: MONOCLONAL ANTIBODY TO A HUMAN CARCINOMA TUMOR ASSOCIATED ANTIGEN

(57) Abstract

A murine monoclonal antibody specific to a particular antigenic determinant on the surface or in the cytoplasm of human carcinoma cells and tissue. A process of making said monoclonal antibodies. A cell line is provided for producing such specific monoclonal antibodies specific for the human carcinoma KC-4 tissue antigen and a method of detecting and measuring said antigen. A method of detecting and diagnosing human carcinomas by selective labelling of said monoclonal antibodies.
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MONOCLONAL ANTIBODY TO A HUMAN CARCINOMA TUMOR ASSOCIATED ANTIGEN

This invention relates to monoclonal antibodies and particularly, to murine monoclonal antibodies which demonstrate reactivity to a specific antigen on the surface or in the cytoplasm of human carcinoma cells and tissue.

The human system involves the production of serum proteins, known as antibodies, by the lymphoid cell series capable of reacting with antigenic determinants which trigger their production. Since the conventional response of the immune system to an antigen with many antigenic determinants is the production of antibodies to each determinant, the antiserum produced is heterologous in nature and polyclonal, or produced by many different cells each producing antibodies to a specific determinant. Antigenic determinants may be referred to as epitopes when more than one occurs on a single molecule and particularly when each elicits an antibody developing, immune response. A single antibody molecule is specific for a unique antigenic determinant or epitope.

Monoclonal antibodies are uniform antibodies directed to a single determinant or epitope on the antigen molecule which may be repeated at several sites of the molecule. Obviously, to produce such monoclonal antibodies in vitro requires selecting a homogeneous antibody having the desired specifications from numerous antibodies elicited in a conventional polyclonal response. The basic technology for in vitro production of homogeneous, highly specific, monoclonal antibodies was developed by Kohler, G. and Milstein, C. (Nature 256:495-497, 1975) known as hybridoma technique. This method involved the immunizing of mice with antigens resulting in the harvesting of antibody-producing cells from those
animals, and fusing these antibody-producing cells with a strain of antibody nonproducing myeloma cells, e.g. plasma cell tumor cells, to produce hybridomas. These hybridomas are robust cells which have all of the *in vitro* survival and growth stamina of the myeloma cell line and antibody producing quality of the B lymphocytes with which it was fused. The hybridomas thus produce monoclonal antibodies and may either be cultured *in vitro* or may be grown as tumors in a host animal. Since each antibody-producing cell produces a single, unique antibody, the monoclonal cultures of hybridomas each produce a homogeneous antibody which may be obtained either from the culture medium of hybridoma cultures grown *in vitro* or from the cells, injected into the peritoneal cavity of mice producing ascitic fluid, or serum of a hybridoma tumor bearing host animal.

Although the general scheme of hybridoma and monoclonal antibody production is well known at this stage of implementation, great care must be exercised in the separation and maintenance of hybridoma cells in culture. Isolated clones have been known to produce antibodies against a subject antigen which differs from clone to clone since antibodies produced by different cells may react with different antigenic determinants on the same molecule. Adequate testing of the resulting antibody or antibody-containing medium, serum or ascitic fluid is essential. It is necessary to characterize the antibody of each clone which contributes to the complexity of producing monoclonal antibodies which are to be utilized in both diagnostic and therapeutic applications.

In developing a desired monoclonal antibody, one must identify and locate the antigenic determinant which will elicit a specific antibody to bind with it. Or, conversely, develop several hundred hybridoma clones from fusions performed and exhaustively screen them
against normal and non-normal tissue and different antigens in identifying and defining that clone which produces the antibody with desired binding specificity. According to this invention the antibody produced detects structural differences on cell surface markers associated with the onset of adenocarcinoma and squamous cell carcinoma, the primary types of carcinoma. The primary object of this invention is to create and maintain hybridomas which produce monoclonal antibodies which will bind with such a particular antigenic determinant to achieve this desired functional specificity.

It is known that monoclonal antibodies may be labeled with a selected variety of labels for desired selective usages in detection, diagnostic assays or even therapeutic applications. In each case, the binding of the labelled monoclonal antibody to the determinant site of the antigen will signal detection or delivery of a particular therapeutic agent to the antigenic determinant on the non-normal cell. A further object of this invention is to provide the specific monoclonal antibody suitably labelled for achieving such desired selective usages thereof.

This invention has particular application to achieving identification of carcinoma cells which occur in the specific diseases of adenocarcinoma and squamous cell carcinoma, the primary forms of carcinoma.

Murine monoclonal antibodies specific to a unique antigenic determinant on the surface and in the cytoplasm of human neoplastic tissue are produced. The unique antigenic determinant is designated the "KC-4 antigen" which is capable of eliciting an antibody which binds selectively only to neoplastic carcinoma cells and not to normal human tissues. The unique antigen appears in two forms in carcinoma cells of which only the smaller is expressed in the cell membrane. The first is the larger
form and appears only in the cytoplasm and has a molecular weight of approximately 490,000 daltons (range of 480,000 - 510,000). The second form occurs at higher density expression and is found in both the cytoplasm and membrane of carcinoma cells and has a molecular weight of approximately 438,000 daltons (range of 390,000 - 450,000) determined by subjecting the KC-4 antigen to electrophoresis methodology and comparing movement thereof with market protein molecules of known molecular weight (Towbin, et al Proc. Natl. Acad. Sci. 76:4350-4354, 1979 and Laemmli, U.K. Nature, 227:680, 1970). The monoclonal antibody, called "KC-4" of the invention has useful application in the areas of diagnosis and medical treatment of a plurality of carcinomas by means of selective labels affixed thereto.

The KC-4 monoclonal antibody is particularly useful in its application to binding with the antigenic determinants on and in carcinoma cells which occur in the specific diseases of adenocarcinoma and squamous cell carcinoma regardless of the human organ of origin.

The present invention provides murine monoclonal antibodies specific to a particular antigen on the surface or in the cytoplasm of human carcinoma tissue, such as adenocarcinoma and squamous cell carcinoma. This unique antigen, designated "KC-4 antigen", was developed from human carcinoma tissue involving prostate adenocarcinoma. All monoclonal antibodies having this specificity for the defined "KC-4 antigen" can be referred to as "KC-4".

A Balb/c mouse was inoculated intraperitoneally over a two week period using an initial injection of prostatic adenocarcinoma cells. Two additional injections followed using as an immunogen a crude tumor homogenate from the
same tumor. The spleen of the mouse was perfused four
days following the additional injections to isolate
individual cells. Then, cells of the mouse plasmacytoma
5 cell line, known as Sp2/0-Ag14, were fused with the mouse
splenocytes using a modified Kohler and Milstein
procedure (Nature 256:495-497,1975). Fused cells were
then cultured for 10-14 days in HAT media to develop cell
colonies capable of multiplying in the media. Conditioned
media containing the antibody secreted from each colony
10 was removed and screened for specific activity. Media was
used to stain normal and prostatic adenocarcinoma tissue.
Fused cell colonies exhibiting the desired reactivity were
single cloned and further tested on a variety of normal
and neoplastic tissues including carcinoma.

The cloning procedure for the selected fused cell
colonies, which were KC-4 producing colonies, was
performed in soft agar. Cells were mixed with liquified
agarose and the mixture was plated in well plates and
allowed to solidify. Then, the plates were incubated and
20 monitored, individual clones being harvested between 10 to
14 days. The individual clones were each screened by
immunoperoxidase and immunoflorescent staining of human
tissue and cell lines. Clones producing the desired
antibody were isolated and cloned again in agarose to
further assure stability and monoclonal nature.

The monoclonal antibody "KC-4" demonstrates an
intense membrane and cytoplasmic antigen distribution on
carcinoma cells and gave no specific or positive staining
pattern on normal human tissue.

Reactivity of the KC-4 monoclonal antibody on normal
and neoplastic human tissues was determined using two
methods including biotin/avidin immunoperoxidase and
immunofluorescence staining procedures. Both fixed and
paraffin embedded tissue, frozen sections, fresh tumor
35 cells and cell lines were used to demonstrate tissue
distribution of the specific antigen being identified. A positive result with KC-4 is seen as an intense membrane and/or cytoplasmic cytoplasmic A neoplastic specimen showed positive staining of the majority of tumor cells present. No specific reactivity with normal tissue specimens or normal cells has been observed throughout the screening analyses.

One hundred and four different cases of solid tumors or lung, colon, kidney, breast, stomach, prostate, pancreatic, lymph node ductal, and lymphoma different tumor tissues were tested with the KC-4 antibody. All such cases were heat processed, paraffin prepared tissues. Ninety-four percent of these cases (98/104) were positive. All positive staining appeared only on tumor cells while all normal tissue remained unaffected. The six percent false negative staining was attributed to poorly prepared tissue which destroyed rather than preserved KC-4 expression.

Ninety-two different cases of paraffin embedded normal tissue including spinal cord, breast, uterus, thyroid, tongue, prostate, spleen, adrenal, lung, kidney, gall bladder, heart, lymph node, stomach, colon, liver, brain, testes, thymus, and placenta were tested with the KC-4 antibody. All 92 cases were heat processed, paraffin prepared tissues. Only 15.2% (14/92) demonstrated some staining. In all of these positives, the staining was attributed to normally occurring artifacts found in these tissues. The greatest amount of non-specific staining of the normal tissue was in breast, kidney, and stomach tissue. The staining in the breast tissue was found in the alveolar cells of the glands. This is a common finding and is considered to be nonspecific on the antibody. The convoluted distal tubules picked up some staining in the kidneys. This is seen with almost all antibodies and is non-specific in origin. Mucous picks up
the stain with most antibodies and this is the case with
the normal stomach tissue and KC-4. This staining is
considered non-specific and artifactual.

Thirty-three different normal tissues from prostate,
lung, kidney, liver, lymph node, spleen, colon, thymus,
breast, gall bladder and stomach were processed by fresh
frozen section and tested with the KC-4 antibody. No heat
was used in processing these specimens. Only 3% (1/33)
demonstrated any positive staining. It should be noted
that frozen tissue sections are more like the fresh tissue
than heat processed, formalin fixed, and paraffin embedded
tissue. Therefore, the difference is percent positive
staining of KC-4 on normal frozen tissue (3%) versus
normal fixed/embedded tissue (15%) is artificfactualy
created in the method of tissue preparation.

Further analyses were conducted on frozen human tumor
tissue of colon, prostate, lung, and breast carcinoma with
KC-4 antibody staining. One hundred percent of the
neoplastic carcinoma tissues were positive with KC-4
i.e., deep cytoplasmic and cell surface specific staining
was observed.

The KC-4 antigen molecule was isolated and identified
as having two forms. The larger of the forms has an
approximate molecular weight of 490,000 daltons (range of
480,000 - 510,000) and occurs only in the cytoplasm of
carcinoma cells. The smaller form has an approximate
molecular weight of 438,000 daltons (range of 390,000
450,000) and occurs in both the cytoplasm and the membrane
of carcinoma cells. This isolation was accomplished by
lysing cells of the HT-17 cell line, derived from a human
breast carcinoma, in distilled water at 1 x 108 cells/ml
employing repeated freezing and thawing. The lysates were
centrifuged at 100,000 x g to prepare a membrane pellet
and a cytoplasm supernatant. The cytoplasm was diluted
1:1 in SDS-PAGE sample buffer. The membranes were
dissolved in SDS-PAGE sample buffer. Both samples were heated to $90^\circ$ for 5 minutes. Subsequently, $23 \times 10^6$ cells equivalent of each sample was run on SDS polyacrylamide (3.5 – 10% gradient) electrophoresis carried out on a discontinuous vertical slab gel according to a modification of the procedure described in Laemmli, U.K. Nature 227, 680, 1980. The internal molecular weight markers were fibrinogen (340,000), fibronectin (440,000), myosin (200,000), beta-galactosidase (116,000), phosphorylase B (92,500), bovine Albumin (66,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and alpha-lactalbumin (14,000). After electrophoresis, the proteins in the acrylamide slab were electrobotted to a sheet of nitrocellulose according to a modification of the procedures described in Towbin (1979) Proc. Natl. Acad. Sci., 76, 4350. The nitrocellulose was then blocked in bovine albumin containing buffer. Monoclonal antibody, KC-4, was then reacted with the nitrocellulose to bind to the specific antigen located on the nitrocellulose. After washing away unbound KC-4 antibody, an anti-mouse immunoglobulin, enzyme conjugate was reacted with the KC-4 antibody bound to the nitrocellulose. After washing away unbound conjugate, enzyme substrate was added and colored bands appear where the KC-4 antigen had migrated.

The "KC-4" monoclonal antibody specifically reactive with the KC-4 antigen was found in two forms. A mouse IgG3 isotype and an IgM as evidenced by its reactivity with a goat anti-mouse IgG3 and IgM antibody and its lack of reactivity with other goat and/or rabbit anti-mouse immunoglobulin isotype specific antibodies.

A sample of both hybrid cell lines capable of producing monoclonal antibodies specific for the KC-4 antigen are on deposit with the American Type Culture Collection and are assigned the Nos. HB 8709 (IgG3) and HB 8710 (IgM).

The availability of homogeneous, highly specific
monoclonal antibodies is an especially valuable tool for diagnostic and therapeutic applications in the detection and treatment of human carcinomas.

As a diagnostic tool, the KC-4 monoclonal antibodies can be brought into contact with a biological sample of human carcinoma cells derived from human neoplasia. Immunological complexes derived between the monoclonal antibody and carcinoma cells in the biological sample can be detected, said complexed cells being monoclonal antibody and human neoplastic cells.

This methodology can also be applied to detect and measure the KC-4 antigen in serum or other liquid biological samples derived from human patients suspected of having human carcinoma or related tumors.

Further, said complexes can be detected by contacting that biological sample of the human carcinoma with a second antibody capable of binding to the KC-4 monoclonal antibody. Said second antibody is labeled with a detectable compound (detector group) selected to enable said complexes to be labelled with said detectible compound when said second antibody binds to said monoclonal antibody specific for the KC-4 antigen. The resulting labelled complex can then be detected. For diagnostic applications, said detector group can be selected from a fluorescent compound, an enzyme which produces absorptive or fluorescent detector group when reacted with a specific substrate, radioactive element, or an electron dense compound. (Goldman, Morris Fluorescent Antibody Methods, Academic Press, New York, 1968; Yoshitake, S. et al. Scand. J. Immunol. 10:1-6, 1979; Hunter, W.M. & Greenwood, F.C. Preparation of iodine 131 labeled growth hormone of high specific activity. Nature 194,495,1962).

Detector groups suitable for this function include fluorescent compounds such as fluorescein, rhodamine,
phycoerythrin, cyamine dyes, and any other compound emitting fluorescence energy. Other categories of detector groups include enzyme substrate products which form fluorescent compounds such as N-methylumbelliferone-B-D-galactosidase or absorptive compounds as DAB (di-aminobenzidine). There are many others in these categories. Radioactive elements which are suitable as detector groups include Iodine-125, Iodine-131, Indium 111, Bismuth-210, and several others of which these are presently the most often used compounds. Electron dense detector groups would include such compounds as gold and ferric chloride, as presently known. Although this approach is predominately employed on \textit{in vitro} diagnostic applications it does not exclude \textit{in vivo} diagnostic or therapeutic application of similarly labeled KC-4 antibody.

The KC-4 monoclonal antibody can be used for detecting carcinoma in a human patient. In this application, KC-4 monoclonal antibody is treated to develop a label thereon capable of producing a detectible signal and infusing said monoclonal antibody into the patient thereby labeling said tumor when the monoclonal antibody binding to the antigenic determinant thereof. Such a detectible label can comprise a radioactive element, a fluorescent compound or other suitable detectible label or compound. This approach is equally suited for \textit{in vitro} diagnostic detection of carcinoma cells on tissues which have been frozen, fixed, or fixed and heat processed with paraffin embedding. Additional \textit{in vitro} applications include the radioimmunoassay or radioimmunometric assay or enzyme immunoassay or nephlemetric detection of KC-4 antigen in serum, plasma, or other liquid based biological samples such as cerebral spinal fluid, urine, and sputum.

For therapeutic treatment with the intent of inhibiting or eliminating human carcinoma in a patient
suspected of having such a tumor, the KC-4 monoclonal antibody of KC-4 conjugated with a suitable toxic agent can be injected into the patient in a controlled protocol of administrations whereby said monoclonal antibody or monoclonal antibody--toxic agent-conjugate can bind to the tumor and effect tumor cell death. Examples of such a toxic agent can be a chemotherapeutic agent, a photo-activated toxic agent or radioactive agent. Examples of such a radioactive agent are Iodine-125, or Bismith-210. Examples of a chemotherapeutic agent would include the alpha chain or A-chain ricin, diphtheria, or whole molecules, cytoxin adriamycin, methyltrexate, and platinium compounds, such as cisplatin. Examples of photo activated toxic agents include infrared dyes, such as in the cyanine family.
Claims:

1. A hybrid cell line which produces a monoclonal antibody specific for the human carcinoma KC-4 tissue antigen.

2. The cell line according to claim 1 characterized in that said antibody-producing cell is derived from the murine genus.

3. The cell line according to claim 1 characterized in that said antibody-producing cells are mouse spleen cells.

4. The cell line according to claim 3 characterized in that said cells are derived from mice immunized with human carcinoma cells.

5. The cell line according to claim 1 characterized in that said cells are derived from a fusion of mouse myeloma cells.

6. The cell line of claim 3 characterized in that the antibody-producing cells were derived from Balb/c mice.

7. The hybrid cell line of any one of claims 1 to 6 characterized by the identifying characteristics of the sample on deposit with the American Type Collection Nos. HB8709 (IgG3) or HB8710 (IgM).

8. The cell line of any one of claims 1 to 7 characterized in that said KC-4 antigen is sited on the surface or in the cytoplasm of the human carcinomas and the antigen further is characterized as having an approximate molecular weight of 438,000 daltons (range of 390,000 - 450,000) and 490,000 daltons (range of 480,000-510,000) as determined by electrophoresis methodology applied to the antigen and comparing the antigen movement with that of known marker proteins of known molecular weights.

9. A monoclonal antibody specific to a particular antigen on the surface or in the cytoplasm of human carcinoma tissue, said antigen being further
characterized in that
  a. it has an approximate molecular weight of 390,000 - 450,000 as determined by carrying out electrophoresis on the antigen and comparing its movement with that of marker proteins of known molecular weight,
  b. it may be expressed in a slightly larger form found only in the cytoplasm having an approximate molecular weight of 490,000 daltons (range of 480,000 - 510,000) as determined by said electrophoresis method,
  c. it is not expressed specifically on normal tissue, and
  d. it is not modulated by a human carcinoma cell line.

10. The monoclonal antibody of claim 9 which is produced by the hybrid cell line having the identifying characteristics of ATCC HB8709 (IgG3) and HB8710 (IgM).

11. A method of detecting or measuring human carcinoma cells derived from human carcinoma in a biological sample, said method characterized by the steps of 1) contacting said biological sample with a monoclonal antibody specific to a particular antigen on the surface of or in the cytoplasm of human carcinoma tissue, said antigen having an approximate molecular weight of 438,000 daltons (range of 390,000 - 450,000) as determined by carrying out electrophoresis on the antigen and comparing its movement with that of marker proteins of known molecular weight capable of being expressed in a slightly larger form found only in the cytoplasm having an approximate molecular weight of 490,000 daltons (range of 480,000 - 510,000) as determined by said electrophoresis method, not expressed specifically on normal human tissue, and
not modulated by a human carcinoma cell line and 2) then
detecting immunological complexes formed between said
monoclonal antibody and cells in said sample, the cells
which are complexed with said antibody being human
5
carcinoma cells.

12. The method of claim 11 wherein said method
is used to detect or measure human carcinoma cells in
a biological sample obtained from a human patient
suspected of having such a carcinoma tumor.

13. The method of claim 12 characterized in that
the step of detecting of said complexes includes the
steps of 1) contacting said biological sample with a
labelled second antibody capable of binding to said
monoclonal antibody, said second antibody being
labelled with a detectable compound such that said
complexes are labelled with said detectable compound
when said second antibody binds to said monoclonal
antibody, and 2) detecting said labelled complexes.

14. The method of claim 13 characterized in that
said detectable compound is a fluorescent compound.

15. The method of claim 13 characterized in that
said detectable compound is an enzyme which produces
said detectable compound.

16. The method of claim 13 characterized in that
said detectable compound is a radioactive element.

17. The method of claim 13 characterized in that
said detectable compound is an electron dense element.

18. A method of detecting human carcinoma in a
patient suspected of having such a tumor, said method
caracterized by the step of infusing KC-4 monoclonal
antibody derivitized with a radioactive element into
said patient, thereby labelling said tumor with the
detectable radioactive element.

19. A method of inhibiting or eliminating human
carcinoma in a patient suspected of having such a
tumor, said method characterized by the step of infusing
KC-4 monoclonal antibody or a said KC-4 monoclonal
antibody-toxic agent conjugate into said patient thereby
contacting said tumor and causing tumor cell death.

20. The method of claim 19 characterized in that
said monoclonal antibody is administered to said
patient in a series of more than one administration.

21. The method of claim 19 characterized in that
said toxic agent is a chemotherapeutic agent.

22. The method of claim 19 characterized in that
said toxic agent is a photoactivated toxic agent.

23. The method of claim 19 characterized in that
said toxic agent is a radioactive agent.

24. The method of claim 23 characterized in that
said radioactive agent is Lodeine 125 or Bismith 210.

25. The method of claim 19 characterized in that
said toxic agent is selected to effect lysing of the
tumor cell with which the antibody binds.

26. The method of claim 25 characterized in that
said toxic agent is selected from either animal
complement used in lysing cells in vivo or in vitro.

27. A murine monoclonal antibody of the mouse
IgG3 or IgM isotype which is specific for the KC-4
antigen.

28. The antibody of claim 27 characterized in
that the KC-4 antigen is selected from the surface
or in the cytoplasm of certain human carcinoma.

29. The antibody of claim 27 characterized in
that it is in detectibly labelled form.

30. The antibody of claim 29 characterized in
that said label is one of the following: a
fluorescent compound, an enzyme which produces said
label, a radioactive or an electron dense element.

31. The antibody of claim 29 characterized in
that said label is a chemotherapeutic, photo-
activated toxic or radioactive agent.
32. A method of detecting the KC-4 antigen of human carcinoma cells by effecting either agglutination of KC-4 monoclonal antibody coated microspheres with carcinoma cells or KC-4 coated fluorescent microspheres satelliting carcinoma cells.

33. A method as described in claim 32 characterized in that said KC-4 antigen is on the surface of the carcinoma cell.

34. A method as described in claim 32 characterized in that said KC antigen is in a liquid biological samples.

35. A method of detecting and measuring KC-4 antigen in a liquid biological samples characterized by the use of KC-4 monoclonal antibody conjugated with a detector group selected from a fluorescent compound, a radioactive element, or enzyme capable of producing a substrate reaction detectible product.

36. A process of making the hybrid cell line as claimed in any one of claims 1 to 8.

37. A process of making the monoclonal antibody as claimed in claims 9 or 10.

38. A method of making a murine monoclonal antibody of the mouse IgG3 or IgM isotype which is specific for the KC-4 antigen.
# INTERNATIONAL SEARCH REPORT

## I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC:

- IPC: C 12 N 5/00; C 12 P 21/00; C 07 K 15/00; C 12 N 15/00;
- IPC: G 01 N 33/574; G 01 N 33/577; A 61 K 49/02; A 61 K 39/395

## II. FIELDS SEARCHED

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## III. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>EP, A, 0087898 (CARLTON MEDICAL PRODUCTS LIMITED) 7 September 1983, see page 4, lines 8-12; page 8, lines 11-19; page 9, lines 1-21; page 11, line 23 - page 12, line 6; page 40, lines 4-13; claims 1, 6</td>
<td>1-17,27-38</td>
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<td>Chemical Abstracts, volume 102, nr. 13, 1 April 1985, Columbus, Ohio, (US) P.W. Andrews et al.: &quot;Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells&quot;, see page 516, abstract nr. 111114</td>
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<td>&amp; Hybridoma 1984, 3(4), 347-61 (Eng)</td>
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  - "E" earlier document published on or after the international filing date
  - "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)
  - "D" document referring to an oral disclosure, use, exhibition or other means

** TY 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
  - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
  - "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.

** A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search: 04 DEC. 1985

International Searching Authority: EUROPEAN PATENT OFFICE

Signature of Authorized Officer: G.L.M. Hofstenberg

Form PCT/ISA/210 (second sheet) (January 1985)
### INTERNATIONAL SEARCH REPORT

**International Application No PCT/US 85/01511**

**I. CLASSIFICATION OF SUBJECT MATTER**

According to International Patent Classification (IPC) or to both National Classification and IPC

- **IPC**: A 61 K 43/00 // (C 12 P 21/00; C 12 R 1:91)

**II. FIELDS SEARCHED**

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**III. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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<td>A</td>
<td>US, A, 4172124 (H. KOPROWSKI) 23 October 1979, see column 1, lines 35-56; claims 1-7,11-14</td>
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**IV. CERTIFICATION**

- **Date of the Actual Completion of the International Search**: 12th November 1985
- **Date of Mailing of this International Search Report**: 04 DEC. 1985

**EUROPEAN PATENT OFFICE**

**Signature of Authorized Officer**: G.L.M. Kruvdenberg
V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

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 ... 18-26... Because they relate to subject matter not required to be searched by this Authority, namely:

SEE RULE PCT 39.1.(iv)

Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

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

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 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 invoice 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 APPLICATION NO. PCT/US 85/01511 (SA 10478)

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 European Patent Office EDP file on 26/11/85.

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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For more details about this annex: see Official Journal of the European Patent Office, No. 12/82.