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(54) **HUMAN MONOCLONAL ANTIBODIES TO THE HEPATITIS B SURFACE ANTIGEN**

MENSCHLICHE MONOKLONALE ANTIKÖRPER GEGEN DAS HEPATITIS B OBERFLÄCHENANTIGEN

ANTICORPS MONOCLONAUX HUMAINS SE FIXANT SUR L'ANTIGENE DE SURFACE DE L'HEPATITE B

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(56) References cited:
EP-A- 0 179 483
EP-A- 0 438 053
WO-A-94/11495
WO-A-94/26784

- **H. MARCUS ET AL.: "Human mouse radiation chimera are capable of mounting a human primary humoral response." BLOOD, vol. 86, no. 1, 1 July 1995, NEW YORK, NY, USA, pages 398-406, XP002044293 cited in the application**
- **G. KÖHLER AND C. MILSTEIN: "Continuous cultures of fused cells secreting antibody of predefined specificity." NATURE, vol. 256, 7 August 1975, LONDON, GB, pages 495-497, XP002044294 cited in the application**
- **I. LUBIN ET AL.: "Engraftment of human peripheral blood lymphocytes in normal strains of mice." BLOOD, vol. 83, no. 8, 15 April 1994, NEW YORK, NY, USA, pages 2368-2381, XP002044295 cited in the application**
- **L. KULOVA ET AL.: "Natural antibodies do not inhibit xenogeneic transplantation of human PBL in lethally irradiated mice." XENOTRANSPLANTATION, vol. 2, no. 1, 1995, pages 8-18, XP002044296**

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(Cont. next page)

- P. EHRLICH ET AL.: "Characterization of human monoclonal antibodies directed against hepatitis B surface antigen." HUMAN ANTIBODIES AND HYBRIDOMAS, vol. 3, January 1992, pages 2-7, XP000573890
- R. EREN ET AL.: "Production of specific human monoclonal antibodies to hepatitis B virus by human lymphocytes engrafted in normal strains of mice." JOURNAL OF HEPATOLOGY, vol. 25, no. suppl. 1, 25 - 29 August 1996, page 80 XP002044297
- R. Eren et al, Hepatology vol. 32, No. 3, 2000, p. 588-596
- E. Galun et al, Hepatology vol. 25, No. 3, 2002, p. 673-679

Description

FIELD OF THE INVENTION

[0001] The present invention concerns hybridoma cell lines which produce human antibodies capable of binding to the hepatitis B virus surface antigen, antibodies produced by the cell lines, and various uses thereof.

BACKGROUND OF THE INVENTION

[0002] Hepatitis B virus (HBV) infection is a major worldwide health problem. Approximately 5% of the world population is infected by HBV and chronically infected patients carry a high risk of developing cirrhosis and hepatocellular carcinoma. (Progress in Hepatitis Research: Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Hepatitis Delta virus (HDV) Ed. O. Crivelli, Sorin Biomedica, 1991).

[0003] The immune response to HBV-encoded antigens includes both a cellular immune response which is active in the elimination of HBV infected cells, as well as a humoral antibody response to viral envelope antigens which contributes to the clearance of circulating virus particles. The dominant cause of viral persistence during HBV infection is the development of a weak antiviral immune response.

[0004] Recombinant HBV vaccines provide a safe and effective means for active immunization against HBV, however, they do not always induce a sufficient and rapid antibody response.

[0005] Interferon-α has been used in the therapy of Hepatitis B infection showing an efficacy of only 30-40% in highly selected patients.

[0006] In addition, passive immunization with human polyclonal anti Hepatitis B antisera has been shown to be effective in delaying and even preventing recurrent HBV infection (Wright, T.L. and Lau, J.Y.N. The Lancet **342**: 1340-1344, (1993)). Such human polyclonal antisera are prepared from pooled plasma of immunized donors. These preparations are very expensive and available in relatively small amounts. Furthermore, pooled plasma may contain contaminated blood samples and thus treatment with such antisera increases the patient's risk to contract other viral infections such as Hepatitis C or HIV.

[0007] An alternative approach for the treatment of HBV infection is the use of monoclonal antibodies (MoAb).

[0008] PCT patent application PCT/NL94/00102 discloses human monoclonal antibodies directed against the Hepatitis B surface antigen which are secreted by the hybridoma cell lines Mab 4-7B and Mab 9H9. The monoclonal antibody secreted by the cell line Mab 4-7B recognizes a linear epitope of HBVsAg and is different from the Mab 9H9 monoclonal antibody which recognizes a conformational epitope. The antibodies are claimed for simultaneous use in the treatment of chronic Hepatitis B infections.

[0009] PCT patent application PCT/US92/09749 discloses human monoclonal antibodies against HBVsAg which are secreted by the hybridoma cell lines PE1-1, ZM1-1, ZM1-2, MD3-4 and LO3-3. The antibodies bind to different HBV epitopes and are used for reducing the level of circulating HBVsAg.

[0010] Japanese Patent Application JP 93066104 discloses a hybridoma of a human lymphocyte cell strain TAW-925 and a human lymphocyte transformed by Epstein-Barr virus. The hybridoma produces a human monoclonal antibody against HBVsAg.

[0011] U.S. Patent Application No. 4,883,752 discloses the preparation of human-derived monoclonal antibody to HBVsAg, by administration of HBVsAg vaccine to humans, recovering their lymphocytes, stimulating the lymphocytes *in vitro* with a non specific stimulator, fusing said cells with a myeloma cell, and selecting for hybridomas which secrete anti HBVsAg antibodies.

[0012] Ichimori *et al*., *Biochem. and Biophysic. Research Communications* **129**(1):26-33, 1985 discloses a hybridoma secreting human anti HBVsAg monoclonal antibodies which recognize the a-determinant of HBVsAg. Later, Ichimori, *et al*., *supra* **142**(3):805-812, 1987 disclosed another hybridoma which stably secretes a human monoclonal antibody against HbsAg.

[0013] The above mentioned antibodies were all developed by *in vitro* immortalization of antibody-producing cells from individuals positive for anti-HBV antibodies.

[0014] A new approach enabling adaptive transfer of human peripheral blood mononuclear cells (PBMC) into lethally irradiated normal strains of mice radioprotected with severe combined immune deficiency (SCID) bone marrow was recently described (Lubin I., *et al*., *Blood*, **83**:2368, 1994). Secondary humoral responses to various recall antigens as well as a primary humoral response to other antigens were shown to be generated effectively in such human/mouse chimeras (Marcus H., *et al*., *Blood*, **86**:398-406, 1995).

SUMMARY OF THE INVENTION

[0015] In accordance with the present invention, it was found that hybridoma cell lines secreting human antibodies

capable of binding to the Hepatitis B surface antigen (HBVsAg) may be obtained using the above mentioned human/mouse chimeras. In accordance with the present invention, human peripheral blood lymphocytes (PBL) from human donors positive for anti HBVsAg antibodies are engrafted into normal strains of mice which were lethally irradiated and radioprotected with SCID bone marrow. After immunization of such chimeric mice with HBVsAg, human cells are obtained from the mice spleens and fused *in vitro* with heteromyeloma cells to generate hybridomas secreting human antibodies having a high affinity and specificity to HBVsAg.

[0016] The antibodies of the present invention are generated by a process for obtaining human monoclonal antibodies (hMoAb) capable of binding to Hepatitis B virus surface antigen (HBVsAg) comprising:

(a) immunizing a chimeric rodent M4 having xenogeneic hematopoietic cells with Hepatitis B surface antigen (HBVsAg) such that xenogeneic antibody-producing cells are produced in said rodent, wherein said rodent M4 is a rodent M1, the hematopoietic cells of which have been substantially destroyed, said rodent M1 having transplanted therein hematopoietic cells derived from a mouse M2 having a hematopoietic deficiency, and xenogeneic hematopoietic cells derived from human M3;
(b) removing and immortalizing said antibody-producing cells;
(c) selecting and cloning the immortalized antibody producing cells producing the antibodies capable of binding to HBVsAg and;
(d) isolating the antibodies produced by the selected, cloned and immortalized antibody producing cells:

[0017] Spleens of the immunized chimeric rodent M4 are removed between 12 and 20 days after human PBL transplantation, preferably at day 14 after transplantation thereof. Cell suspensions are prepared from the spleens and the antibody producing cells obtained from the immunized chimeric rodent M4 are fused preferably with a human-mouse fusion partner such as heteromyeloma by techniques well-known in the art (e.g. Köhler & Milstein, Nature, **256**:495-497, 1975). In order to isolate the antibodies produced by the selected hybridoma cell lines, the hybridoma cell lines are either cultured *in vitro* in a suitable medium wherein the desired monoclonal antibody is recovered from the supernatant or, alternatively, the hybridoma cell lines may be injected intraperitoneally into mice and the antibodies harvested from the malignant ascitis or serum of these mice. The supernatants of the hybridoma cell lines are first screened for production of human IgG antibodies by any of the methods known in the art such as enzyme linked immunosorbent assay (ELISA) or radioimmuno assay (RIA). Hybridomas testing positive for human IgG are then further screened for production of anti HBVsAg antibodies by their capability to bind to HBVsAg.

[0018] The M1 rodent is a rodent conventionally used as a laboratory animal, such as a rat or a mouse.

[0019] The mouse M2 may have any hematopoietic deficiency including genetic hematopoietic deficiencies as well as induced hematopoietic deficiencies. Non limiting examples of hematopoietic deficiencies include SCID, Bg, Nu, Xid or mice having any combination of the above mentioned hematopoietic deficiencies. In addition, the hematopoietic deficiency may also be a result of gene deletion or transgenic mice may be used.

[0020] The hematopoietic cells derived from the donor mouse M2 are bone marrow cells either untreated or depleted of T cells. Other suitable sources of hematopoietic cells which may also be used include, for example, spleen cells, fetal liver cells or peripheral blood cells.

[0021] The xenogeneic hematopoietic cells derived from the human M3 are PBL cells but may also be derived from any suitable source of human hematopoietic cells such as bone marrow cells, cord blood cells, thymus, spleen or lymphnode cells, etc.

[0022] The rodent M1 may be a mouse or rat, the mouse M2 may be a SCID mouse and the xenogeneic hematopoietic cells derived from the human M3 may be PBL from a human M3 which has already been exposed to the HBVsAg either spontaneously as a result of a prior infection or induced following vaccination. Such humans will have a relatively high titer of anti HBVsAg antibodies as compared to individuals which have never been infected with HBV and, therefore, when PBLs from such donors are used as M3 donor cells in accordance with the present invention, the immunization of the M4 chimeric mouse with HBVsAg will elicit a secondary immune response of the transplanted human PBL in the M4 chimeric mouse. A human donor M3, for example, is such which tested negative for the HB virus but shows a high titer of antibodies against HBVsAg. Such PBL from the human M3 donor may be obtained either by whole blood donation or by leukophoresis.

The HBVsAg used for immunizing the chimeric rodent M4 in accordance with the invention is, for example, a Hepatitis B virus vaccine containing the purified major surface antigen of the virus prepared by recombinant DNA technology (Engerix ™-B, SIB Biological (Rixensart, Belgium)).

[0023] The present invention is directed to hybridoma cell lines as defined in Claim 2 which produce human monoclonal antibodies capable of binding to HBVsAg, as well as to human monoclonal antibodies capable of binding to HBVsAg as defined in claim 1 and fragments thereof substantially maintaining the antigen binding characteristics of the whole antibody. Such fragments may be, for example, Fab or $F(ab)_2$ fragments obtained by digestion of the whole antibody with various enzymes as known and described extensively in the art. The antigenic characteristics of an

antibody are determined by testing the binding of an antibody to a certain antigenic determinant using standard assays such as RIA, ELISA or FACS analysis.

[0024] Typically, the human monoclonal antibodies of the present invention have a relatively high affinity to HBVsAg being in the range of about 10^{-9}M to about 10^{-10}M as determined in a competitive ELISA.

[0025] In accordance with a specific embodiment of the present invention there is provided a hybridoma cell line designated herein as "*19.79.5*" which was deposited on May 22, 1996, in the European Collection of Cell Cultures (ECACC, CAMR, Salisbury, Wiltshire, SP40JG, U.K.) under Accession No. 96052168. Anti HBVsAg human monoclonal antibodies secreted by the above hybridoma cell line and designated herein as *"Ab19.79.5"* are also provided as well as fragments thereof retaining the antigen binding characteristics of the antibodies.

[0026] Further aspects of the present invention are various diagnostic, prophylactic and therapeutic uses of the human anti HBVsAg monoclonal antibodies. In accordance with this aspect of the invention, pharmaceutical compositions comprising the human anti HBVsAg monoclonal antibodies may be used for the treatment of chronic Hepatitis B patients by administering to such a patient a therapeutically effective amount of the monoclonal antibody or portion thereof capable of binding to the HBVsAg being an amount effective in alleviating the symptoms of the HBV infection or reducing the number of circulating viral particles in an individual.

Such pharmaceutical compositions may comprise one or more antibodies of the invention. In addition to the antibodies of the invention the pharmaceutical compositions may optionally also comprise a carrier selected from any of the carriers known in the art. One example of such a carrier is a liposome. The pharmaceutical compositions of the invention may also comprise various diluents and adjuvants known per se.

The compositions of the invention may be administered by a variety of administration modes including parenterally, orally etc.

Compositions comprising the antibodies of the invention, as described above, may be administered in combination with other anti viral agents. Such agents may include, as non limiting examples: Interferons, anti HB monoclonal antibodies, anti HB polyclonal antibodies, nucleoside analogues, and inhibitors of DNA polymerase. In the case of such a combination therapy the antibodies may be given simultaneously with the anti viral agent or sequentially either before or after treatment with the anti viral agent.

The pharmaceutical compositions of the invention may also be used, for example, for immunization of new born babies against HBV infections or for immunization of liver transplantation patients to eliminate possible recurrent HBV infections in such patients.

By a further embodiment, the antibodies of the invention may also be used in a method for the diagnosis of HBV infections in an individual by obtaining a body fluid sample from the tested individual which may be a blood sample, a lymph sample or any other body fluid sample and contacting the body fluid sample with a human anti HBVsAG antibody of the invention under conditions enabling the formation of antibody-antigen complexes. The level of such complexes is then determined by methods known in the art, a level significantly higher than that formed in a control sample is indicating an HBV infection in the tested individual. In the same manner, the specific antigen bound by the antibodies of the invention may also be used for diagnosis.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] **Fig. 1** is a graphic representation showing the amount of total human Ig (mg/ml) and the amount of specific anti HBs antibodies (mU/ml) in the sera of irradiated mice which were radioprotected with SCID bone marrow (chimeric mice). PBL+Engerix: the chimeric mice were further transplanted with human PBL from donors positive for anti HBs antibodies, and vaccinated with Engerix B in an aluminum hydroxide adjuvant (alum).

PBL+Alum: the chimeric mice were further transplanted with human PBL from donors positive for anti HBs antibodies, and vaccinated with Alum alone (no Engerix B).

SCID-BM+Engerix: the chimeric mice were vaccinated with Engerix B (no transplantation of human PBL).

SCID-BM+Alum: the chimeric mice were vaccinated with Alum (no human PBL and no Engerix B).

The black line represents the initial level of anti HBs antibodies in the serum of the human PBL donor.

[0028] **Fig. 2** is a graphic representation showing the specific activity, i.e. the levels of anti HBVs antibodies per mg of human Ig in the sera of human donors (A-D, black columns) and the specific activity in the sera of chimeric mice transplanted respectively with human PBL of said donors (A-D, striped columns).

[0029] **Fig. 3** is a graphic representation showing the time response curve of anti HBs antibodies specific activity (mU/mg) in sera of chimeric mice (dotted line). The black columns represent the level of total human Ig (mg/ml), and the striped columns represent the level of specific anti HBs antibodies (mU/ml).

[0030] **Fig. 4** is a graphic representation showing competitive inhibition of binding of anti HBs antibodies to HBs particles. The extent of binding was measured by ELISA using a horseradish peroxidase labeled anti human IgG secondary antibody. The anti HBs antibodies were diluted as indicated in the graph in medium (empty squares) or in 0.5 μg/ml HBs particles (black squares).

[0031] **Fig. 5** is a photograph showing Hepatitis B infected liver sections stained with anti HBVs antibodies. All sections were stained with a "*secondary*" antibody, i.e. goat anti human Ig conjugated to biotin.

A - negative control. No first antibody.
positive control. First antibody - mouse anti HB antibody and a secondary anti-mouse Ig.
C - staining with anti HBs antibody No. 19.79.5.
D - staining with anti HBs antibody No. 18.5.1013.

[0032] **Fig. 6** is a schematic representation of the binding of Ab 19.79.5 to a set of 15 well characterized HBsAg types. The y axis represents optical density units. The x axis represents different HBsAg types.

[0033] Fig. 7 is a graphic representation of the percentage of HBV infected animals at days 11 and 18 in the untreated group and Ab 18.5.1013 treated group (in the inhibition model).

[0034] **Fig. 8** is a graphic representation of the percentage of HBV infected animals at days 10 and 17 in the untreated group and Ab 19.79.5 treated group (in the combined prophylaxis/inhibition model).

[0035] **Fig. 9** is a graphic representation of the percentage of HBV infected animals at days 11 and 19 in the untreated group and Ab 19.79.5 treated group (in the combined inhibition/treatment model).

[0036] **Fig. 10** Nucleic acid sequence and corresponding amino acid sequence of the light chain of the variable domain of Ab 19.79.5.

[0037] **Fig. 11** Nucleic acid sequence and corresponding amino acid sequence of the heavy chain of the variable domain of Ab 19.79.5.

[0038] Reference will now be made to the following Examples which are provided by way of illustration and are not intended to be limiting to the present invention.

EXAMPLES

MATERIALS AND METHODS

Mice:

[0039] Animals used were 6-10 weeks old. BALB/c mice were obtained from Harlan (Weizmann Institute Animal Breeding Center (Rehovot, Israel)), SCID/NOD mice from the Weizmann Institute Animal Breeding Center (Rehovot, Israel). All mice were fed sterile food and acid water containing cyprofloxacin (20 μg/ml) (Bayer, Leverkusen, Germany). Whenever necessary, mice were injected daily with 1 mg Fortum i.p. for five days post BMT (Glaxo Operations UK, Greenford, England).

Conditioning Regimens:

[0040] BALB/c mice were exposed to total body irradiation (TBI), from a gamma beam 150-A 60Co source (produced by the Atomic Energy of Canada, Kanata, Ontario) with F.S.D of 75 cm and a dose rate of 0.7 Gy/min, with 4 Gy followed 3 days later by 10-11 Gy (split dose).

Preparation and Transplantation of Bone Marrow Cells:

[0041] The femoral and tibial bones were removed from mice and homogenized in a sterilized 50 ml Omni-Mixer stainless steel chamber (Omni-Mixer Homogenizer, Model No. 17106, OMNI International, Waterbury, CT. USA). Recipient mice were injected i.v. with 4-6 x 10^6 of SCID/NOD bone marrow cells (in 0.2 ml PBS) immediately after irradiation.

Transplantation of Peripheral Blood Lymphocytes:

[0042] Peripheral blood lymphocytes (PBL) were obtained after informed consent by leukophoresis from donors positive for HBs antibodies and negative for HBV. PBLs were washed twice, counted and resuspended in PBS to the desired cell concentration.

[0043] 100 x 10^6 human PBL were injected intraperitoneally (i.p.) into recipient mice, conditioned as described above. Control mice did not receive human PBL.

Immunization of the Chimeric Animals:

[0044] Mice were immunized once with Hepatitis B vaccine (Engerix™-B; SB Biologicals, Rixensart, Belgium) administered i.p. together with the PBL.

Cell and Plasma Collection from Human Mouse Chimera:

[0045] Animals were bled from the retro-orbital vein using heparin-coated glass capillaries. Plasma was kept for human-Ig determination. Spleens were removed after the animals were sacrificed by cervical dislocation, cut into pieces and pressed through stainless steel sieves to make cell suspensions in PBS.

Cell Fusion:

[0046] Cells were mixed with the human-mouse heteromyeloma HMMA2.11TG/0 (Posner *et al. Hybridoma*, **6**: 611-625, 1987) at 3:1 ratio. Fusion was performed with 50% (w/v) PEG 1500 (Boehringer Manheim GmbH) in a standard procedure. Fused cells were seeded at a concentration of 30000 cells/well in 96-well U-bottom microtiter plates (Nunc, Denmark) in complete medium containing HAT-supplement (1x) (Biological Industries, Beit Haemek, Israel). Cells were fed with fresh HAT-medium a week later. Two weeks after fusion supernatants were harvested for ELISA and medium was replaced with fresh HT-medium.

[0047] Hybridoma cultures secreting specific anti-HBs Ig were cloned at 0.5 cell/well in 96-well U-bottom microtiter plates.

Determination of Human Immunoglobulin:

[0048] Sera were tested for antigen specific and total human Ig. Total human Ig was quantified by sandwich ELISA using goat $F(ab)_2$-purified anti-human IgG+IgM+IgA (Zymed Laboratories, San Francisco, CA) as the capture agent and peroxidase-conjugated purified goat anti-human (Zymed Laboratories) as the detection reagent. Human serum of known immunoglobulin concentration was used as the standard (Sigma, Rehovot,Israel). Microplates (Nunc, Roskilde, Denmark) pre-coated with the capture reagent (2.5 ug/ml, 50 ul/well) and blocked with 1% BSA were incubated overnight at 4°C with dilutions of plasma from 1:20000 to 1:640000, or the standard from 0.2 to 0.06 ug/ml, then washed 5 times with PBS-Tween solution. The detection reagent was added and the plates were incubated for 1h at 37°C, then washed again 3 times. Fresh substrate solution (TMB, Sigma) was added and, after peroxidase-catalyzed color development, the reaction was stopped by addition of 10% sulfuric acid. Absorbance at 450 nm was quantified on an ELISA reader (Dynatech, Port Guernsey, Channel Islands, UK).

[0049] Concentration of antigen-specific human antibodies in mice sera was determined by HBsAb EIA kit (ZER, Jerusalem, Israel).

[0050] Human antibodies in hybridoma supernatants were determined by overnight incubation of supernatants on goat anti-human IgG+A+M (Zymed) coated plates, with goat anti-human IgG-peroxidase conjugate as the secondary reagent.

[0051] Antigen-specific antibodies in hybridoma supernatants were determined as above using Hbs antigen coated plates.

Determination of Human IgG Subclasses:

[0052] Human IgG subclasses were determined by sandwich ELISA using goat $F(ab)_2$-purified anti-human IgG+IgM+IgA (Zymed Laboratories, San Francisco, CA) coated plates and Hbs antigen coated plates. Mouse anti-human IgG subclasses (Sigma) were used as secondary antibody and peroxidase-conjugated purified goat anti-human antibody (Zymed Laboratories) as the detection reagent.

Statistical Analysis:

[0053] Statistical analysis was performed using the Stat View II program (Abacus Concepts, Inc., Berkeley, CA) on a Mackintosh Quadra 605 or Microsoft Excel 5.0 (Microsoft) on a 486 DX2 PC compatible. Student t-test, Anova correlation and regression analysis were utilized to calculate probability (p) and correlation coefficient (r) values. Results are presented as mean ± standard error.

Affinity Constant Measurements:

[0054] Determination of affinity constants (K_D) of the different anti-HBs antibodies to ad antigen (Chemicon Cat. No. AG 850) in solution were performed according to Friguet *et al*. (*Journal of Immunological Methods*, **77**:305-319, 1985). The antigen at various concentrations ($3.5x10^{-10}$M to $1.4x10^{-9}$M) was first incubated in solution with a constant amount of antibody ($3.4x10^{-11}$M), in 0.1 M sodium phosphate buffer containing 2 mM EDTA and 10 mg/ml BSA, pH 7.8 (medium buffer). After o.n. incubation at 20 °C the concentration of free antibody was determined by an indirect ELISA. A volume of 300ul of each mixture were transferred and incubated for 2h at 20 °C into the wells of a microtiter plate (Nunc) previously coated with Ad (50 µl/well at 1 µg/ml in 0.1 M $NaHCO_3$ buffer, pH 9.6 for 2 h at 37°C). After washing with PBS containing 0.04% Tween 20, the bound antibodies were detected by adding HRP-$F(ab')_2$ goat anti human IgG (Zymed) diluted 1:3000 with medium buffer, 50 µl/well 2 h at 20°C. The plate was developed with TMB chromogen (Sigma T-3405 tablets) 50 µl/well, the reaction stopped with 10% H_2SO_4 50 µl/well and the plate read in an ELISA reader at 450 nm. The conditions were chosen so that the resulting f values (see Friguet *et al*.) were around 0.1. The antibody concentration used was deduced from an ELISA calibration done on the same plate. The affinity constant K_D was calculated from the relevant Scatchard plot.

Inhibition Assays:

[0055] The inhibition assay was performed in microtiter plates coated with HBs particles (2 µg/ml in PBS). The plate was blocked with 3% BSA in PBS. Hybridoma supernatants containing anti HBs antibodies were serially diluted. 50 µl of each dilution were added to the coated microtiter wells. Subsequently, 50 µl of HBs particles (ad/ay, 0.5 µl/ml in PBS) or PBS alone were added to each well. The plates were incubated overnight at room temperature in a humid chamber and washed 5 times with PBS-Tween. Next, 50 µl of goat anti human IgG conjugated to HRP (diluted 1:5000 in PBS) were added to each well. After a 4 hour incubation at room temperature in a humid chamber the plates were washed 5 times with PBS-Tween, and TMB was added to each well. Results were read using an ELISA reader, at a wavelength of 450 nm.

Immunohistostaining:

[0056] HBV positive liver fragment was fixed in 4% neutral buffered formaldehyde for 24 h and then embedded in paraffin using routine procedures. Sections of 4 µm thickness were cut from paraffin blocks and mounted on polylysine-coated slides. After deparaffinization and peroxidase quenching staining was performed using our monoclonal Human anti-HBs Protein A-purified antibodies followed by biotinylated goat anti-Human IgG (H + L) (Zymed, San Francisco, CA) using a Histostain-SPTM kit (Zymed) according to the manufacture's recommendation. Control slides without using the 1st human anti-HBs antibody were stained in parallel.

Sequence analysis:

[0057] Total RNA was isolated from 10 x 10^6 hybridoma cells with RNAsol B reagent (TEL-TEX, Inc. Friendswood, Texas). cDNA was prepared from 10µg of total RNA with reverse transcriptase and oligo dT (Promega, Madison, WI) according to standard procedures. PCR was performed on 1/50 of the RT reaction mixture with V_H, V_λ or V_κ 5' leader primers and 3' primers corresponding to the human constant region. The PCR fragments were cloned into pGEM-T vector (Promega). The inserts were sequenced using an ABI 377 sequencer. Sequences were analyzed by comparison to GenBank and by alignment to Kabat sequences (Kabat et al. 1991, Sequences of proteins of immunological interest (5^{th} Ed.) U.S. Dept. of Health and Human Services, National Institutes of Health, Bethesda, MD).

Example 1: Production of human anti HBs antibodies in chimeric mice

[0058] Human peripheral blood lymphocytes (PBL) from donors positive for anti HBs antibodies were implanted intraperitoneally into irradiated BALB/c mice which were radioprotected by transplantation of bone marrow from SCID mice. These chimeric mice were immunized with Hepatitis B vaccine (Engerix B) to induce a secondary immune response. The production of specific anti HBs antibodies along with total human Ig secretion was measured in mice sera. Fig. 1 shows levels of total human Ig and specific anti HBs antibodies in mice sera 14 days after transplantation of human PBL. Although the levels of human Ig secreted are similar in immunized and control mice, a strong specific immune response develops in mice vaccinated with hepatitis B vaccine as compared to the control group. Comparison of the levels of specific human antibodies produced in response to the antigen in immunized mice to their levels in the donors sera, indicates a 5-10 fold increase in the mice. Moreover, the specific activity measured in mice sera, i.e. the levels of anti HBs specific antibodies per mg of human Ig secreted, is 102-104 fold higher than the specific activity

observed in the donor. This increase demonstrates a very high amplification of anti HBs antibody production in response to the antigen in the chimeric mice (Fig. 2). Production of human antibodies is detectable 10 days after immunization and reaches a plateau after three weeks. The specific activity is high at day 13 after immunization and decreases thereafter (due to increase in total human Ig secretion) (Fig. 3).

Example 2: Preparation and characterization of human monoclonal antibodies against HBs

[0059] Human B cells harvested from mice spleens two weeks after immunization were fused to human- mouse heteromyeloma cells (Posner *et al. Supra*). Hybridoma cells were tested for their growth rate, total Ig secretion and specific antibody production. Control fusion experiments were performed on the donor PBL that were activated in vitro with PWM and HBVsAg. Fusion frequencies in different experiments ranged from 0.9 -5 x 10-5. Most of the growing hybridoma clones secreted human Ig of which 0.1-4 % produced specific human anti HBs antibodies. Anti-HBs secreting hybridoma cells derived from chimeric mice spleens were compared to those obtained from fusion of the donors *in vitro* activated PBL in terms of Ig type and stability as seen in Table 1 below. The majority of the hybridomas from chimeric mice were found to be IgG type and all were stable for more than 12 months. In contrast, hybridomas derived from donor PBL were mostly unstable, only one clone has been stable for more than 12 months. Two stable hybridoma clones that secreted specific human anti HBs monoclonal antibodies were characterized. As seen in Table 2 below, these antibodies were purified on a protein A column as well as on an anti human Ig - agarose column and were both found to be of IgG1 subclass. Affinity constants ranged from $1.3x10^{-9}$ M to $6x10^{-9}$ M as tested by competitive ELISA. Specificity was tested by competitive inhibition assay using HB surface antigen of the ad-ay (1:1) subtype (Fig. 4). Fig. 5 shows specific binding of the human MoAbs of the invention to HBV by staining human liver fragments infected with HBV.

The gene encoding the variable region of Ab 19.79.5 was isolated, fully sequenced, and its subgroups and CDRs were determined.

The antibody has a full human Ig gene sequence as determined by alignment to GeneBank sequences and Kabat protein sequences. Fig. 10 shows the nucleotide sequence of the cDNA encoding the light chain of the variable region of Ab 19.79.5 and its corresponding amino acid sequence (Sequence identification Nos. 1 and 3). Fig. 11 shows the nucleotide sequence of the cDNA encoding the heavy chain of the variable region of Ab 19.79.5 and its corresponding amino acid sequence (Sequence identification nos. 2 and 4).

The sequence data revealed that the variable region of Ab 19.79.5 consists of the subgroups V_{H3}, J_{H2}, $V_{\lambda 3}$ and $J_{\lambda 3}$.

HBV genomes are classified into six groups A to F, based on the degree of similarity in their nucleotide sequences. The genetic variability of HBV is further reflected in the occurrence of different serotypes of HBsAg. The common determinant '*a*' and two pairs of mutually exclusive determinants '*d*/*y*' and '*w*/*r*' enable the distinction of four major subtypes of HBsAg: *adw, adr, ayw* and *ayr*.

Additional determinants designated subdeterminants of *w* (*w*1 to *w*4) have allowed the definition of four serotypes of *ayw* (*ayw*1-4) and two serotypes of *adw*, i.e. *adw*2 and *adw*4. Additional subtype variation is added by the *q* determinant, which is present on almost all subtypes. Its absence is marked by a '*q*-' sign.

The kind of HBV serotypes recognized by Ab 19.79.5 was examined using a set of 15 different HBsAg types (Norder et al., 1992, Journal of General Virology, 73, 3141; Magnius and Norder, 1995, Intervirology, 38, 24-34). As can be seen in Fig. 6, Ab 19.79.5 has a complex recognition pattern of the different HBsAg serotypes.

Example 3: Biological activity of human monoclonal antibodies against HBs.

[0060] The biological activity of Ab 19.79.5 and Ab 18.5.1013 (the latter antibody not being part of the claimed invention) was characterized using the following HBV animal model: a mouse was treated so as to allow the stable engraftment of human liver fragments. The treatment included intensive irradiation followed by transplantation of SCID (severe combined immunodeficiency) mice bone marrow. Viral infection of human liver fragments was performed ex-vivo using HBV positive human serum (EP 699 235).

The animal model was used in three different modes representing various potential uses of the antibodies: inhibition of infection mode, combined prophylaxis/inhibition mode and combined inhibition/treatment.

1. Inhibition mode - This model demonstrates the ability to use the antibody to inhibit liver infection by HBV. The experiment shown in Fig. 7 is meant for illustrative purposes only. HBV positive human serum was preincubated with Ab 18.5.1013, followed by standard ex-vivo liver infection. HBV-DNA in mice sera was tested 11 and 18 days after transplantation. As seen in Fig. 7 there was a significant reduction in the percentage of infected animals in the antibody treated group as compared to the untreated group.
2. Combined prophylaxis/inhibition mode - This model represents liver transplantation. In this model mice were treated with Ab 19.79.5(10 I.U./mouse) three days before liver transplantation followed by transplantation of human

liver fragments which were ex vivo infected with HBV in the presence of Ab 19.79.5 (100 I.U.). HBV DNA was tested in mice sera 10 and 17 days after transplantation. As can be seen in Fig. 8, there was a significant reduction in the percentage of infected animals in the treated group compared to the control group.

3. Combined inhibition/treatment mode - a) HBV positive human serum was preincubated with Ab 19.79.5 followed by standard *ex vivo* liver infection. b) Mice were treated with Ab 19.79.5 at days 0 and 7 post transplantation. HBV DNA in mice sera was tested on days 11 and 19. As can be seen in Fig. 9, the percentage of infected animals in the Ab 19.79.5 treated group was significantly reduced but rebounded about two weeks after the treatment was stopped.

Example 4: Combination therapy of human monoclonal antibodies against HBs and an anti viral agent

[0061] Using the HBV model described above, mice were treated with an anti viral drug (a nucleoside analogue, 0.5 mg/mouse/day) at days 17-20 post transplantation. A group of mice was further treated with the human monoclonal antibodies of the invention at days 19 and 20. The presence of HBV DNA in mice sera was tested on days 21 and 27.

Table 1

Stability	Anti-HBs Secretors		Source of Hybridoma Cells
	IgM	IgG	
1 stable for > 10 months 47 unstable	25 (52%)	23 (48%)	*In Vitro* Activated PBL
6 stable for > 10 months 3 unstable	3 (33%)	6 (67%)	Chimeric Mouse Splenocytes

Table 2

Kd (M)	Production mg/10^5 cells/day	Type	Clone
6.1×10^{-9}	10.3	IgG1 V1	18.5.1013
1.62×10^{-9}	5.8	IgG1 V1	19.79.5

Claims

1. A human monoclonal antibody being selected from the group consisting of:

   (a) the monoclonal antibody which is secreted by the hybridoma cell line deposited in the European Collection of Cell Cultures (ECACC) under Accession No. 96052168;
   (b) fragments of the antibody of (a) which substantially retain the antigen binding characteristics of the whole antibody;
   (c) a monoclonal antibody encoded by nucleic acid sequences comprising the nucleic acid sequences as shown in Fig. 10 and Fig. 11; and
   (d) a monoclonal antibody comprising the amino add sequences as shown in Fig. 10 and Fig. 11.

2. A hybridoma cell line deposited at the ECACC under Accession No. 96052168.

3. A pharmaceutical composition comprising at least one antibody of claim 1 and optionally a pharmaceutical acceptable carrier.

4. The pharmaceutical composition of claim 3 further comprising at least one other active ingredient being an anti viral agent.

5. Use of the antibody of claim 1 for the preparation of a pharmaceutical composition for the prevention and/or treatment of HBV infections.

6. Use of an antibody of claim 1 in combination with an anti viral agent for the preparation of a pharmaceutical composition for the prevention and/or treatment of HBV infection.

7. The pharmaceutical composition of claim 4 or the use of claim 6, wherein said anti viral agent is selected from the group consisting of interferons, anti HB polyclonal antibodies, nucleoside analogues and inhibitors of DNA polymerase.

8. A method for the diagnosis of HBV infections in a body fluid sample comprising:

(a) contacting said sample with the antibody of claim 1 under conditions enabling the formation of antibody-antigen complexes;
(b) determining the level of antibody-antigen complexes formed;

wherein a level significantly higher than that formed in a control sample indicates an HBV infection in the tested body fluid sample.

Patentansprüche

1. Menschlicher monoclonaler Antikörper, ausgewählt aus der Gruppe bestehend aus:

(a) dem monoclonalen Antikörper, der von der Hybridomzelllinie sezerniert wird, die bei der Europäischen Sammlung von Zellkulturen (European Collection of Cell Cultures (ECACC)) unter der Hinterlegungsnummer 96052168 hinterlegt wurde;
(b) Fragmenten des Antikörpers aus (a), die im Wesentlichen die Antigenbindungsmerkmale des gesamten Antikörpers beibehalten;
(c) einem monoclonalen Antiköper, codiert von den Nucleinsäuresequenzen, die die in den Figuren 10 und 11 gezeigten Nuleinsäuresequenzen umfassen; und
(d) einem monoclonalen Antikörper, der die in den Figuren 10 und 11 gezeigten Aminosäuresequenzen umfasst.

2. Hybridomzelllinie, die bei der ECACC unter der Hinterlegungsnummer 96052168 hinterlegt wurde.

3. Arzneimittel, das mindestens einen Antikörper nach Anspruch 1 und gegebenenfalls einen pharmazeutisch verträglichen Träger umfasst.

4. Arzneimittel nach Anspruch 3, das weiterhin mindestens einen weiteren Wirkstoff umfasst, der ein antiviraler Stoff ist.

5. Verwendung des Antiköpers nach Anspruch 1 für die Herstellung eines Arzneimittels zur Vorbeugung und/oder Behandlung von Infektionen mit HBV.

6. Verwendung eines Antikörpers nach Anspruch 1 in Verbindung mit einem antiviralen Stoff für die Herstellung eines Arzneimittels zur Vorbeugung und/oder Behandlung einer Infektion mit HBV.

7. Arzneimittel nach Anspruch 4 oder Verwendung nach Anspruch 6, wobei der antivirale Stoff ausgewählt ist aus der Gruppe bestehend aus Interferonen, polyclonalen Anti-HB-Antikörpern, Nucleosidanaloga und Inhibitoren von DNA-Polymerase.

8. Verfahren zur Diagnose von Infektionen mit HBV in einer Probe aus Körperflüssigkeit, umfassend:

(a) Inkontaktbringen der Probe mit dem Antikörper nach Anspruch 1 unter Bedingungen, die die Bildung von Antikörper-Antigen-Komplexen ermöglichen;
(b) Bestimmen des Spiegels der gebildeten Antikörper-Antigen-Komplexe;

wobei ein Spiegel, der wesentlich höher ist als der, der sich in einer Kontrollprobe gebildet hat, anzeigt, daß die untersuchte Probe aus Körperflüssigkeit mit HBV infiziert ist.

Revendications

1. Anticorps monoclonal humain étant choisi dans le groupe formé par :

   (a) l'anticorps monoclonal qui est sécrété par la lignée cellulaire d'hybridome déposée à l'European Collection of Cell Cultures (ECACC) sous le n° d'accès 96052168 ;
   (b) des fragments de l'anticorps de (a) qui conservent essentiellement les caractéristiques de liaison à l'antigène de l'anticorps entier ;
   (c) un anticorps monoclonal codé par des séquences d'acides nucléiques comprenant les séquences d'acides nucléiques telles que présentées dans la figure 10 et la figure 11 ; et
   (d) un anticorps monoclonal comprenant les séquences d'acides aminés telles que présentées dans la figure 10 et la figure 11.

2. Lignée cellulaire d'hybridome déposée à l'ECACC sous le n° d'accès 96052168.

3. Composition pharmaceutique comprenant au moins un anticorps selon la revendication 1, et éventuellement un véhicule acceptable sur le plan pharmaceutique.

4. Composition pharmaceutique selon la revendication 3, comprenant de plus au moins un autre principe actif qui est un agent antiviral.

5. Utilisation de l'anticorps selon la revendication 1, pour la préparation d'une composition pharmaceutique destinée à la prévention et/ou au traitement d'infections par HBV.

6. Utilisation de l'anticorps selon la revendication 1, en combinaison avec un agent antiviral pour la préparation d'une composition pharmaceutique destinée à la prévention et/ou au traitement d'une infection par HBV.

7. Composition pharmaceutique selon la revendication 4, ou utilisation selon la revendication 6, dans laquelle ledit agent antiviral est choisi dans le groupe formé par des interférons, des anticorps polyclonaux anti-HB, des analogues de nucléoside et des inhibiteurs de l'ADN polymérase.

8. Procédé destiné au diagnostic d'infections par HBV dans un échantillon de liquide corporel comprenant les étapes consistant :

   (a) à mettre en contact ledit échantillon avec l'anticorps selon la revendication 1 dans des conditions permettant la formation de complexes anticorps-antigène ;
   (b) à déterminer le taux de complexes anticorps-antigène formés ;

   dans lequel un taux significativement supérieur à celui formé dans un échantillon témoin indique une infection par HBV dans l'échantillon de liquide corporel testé.

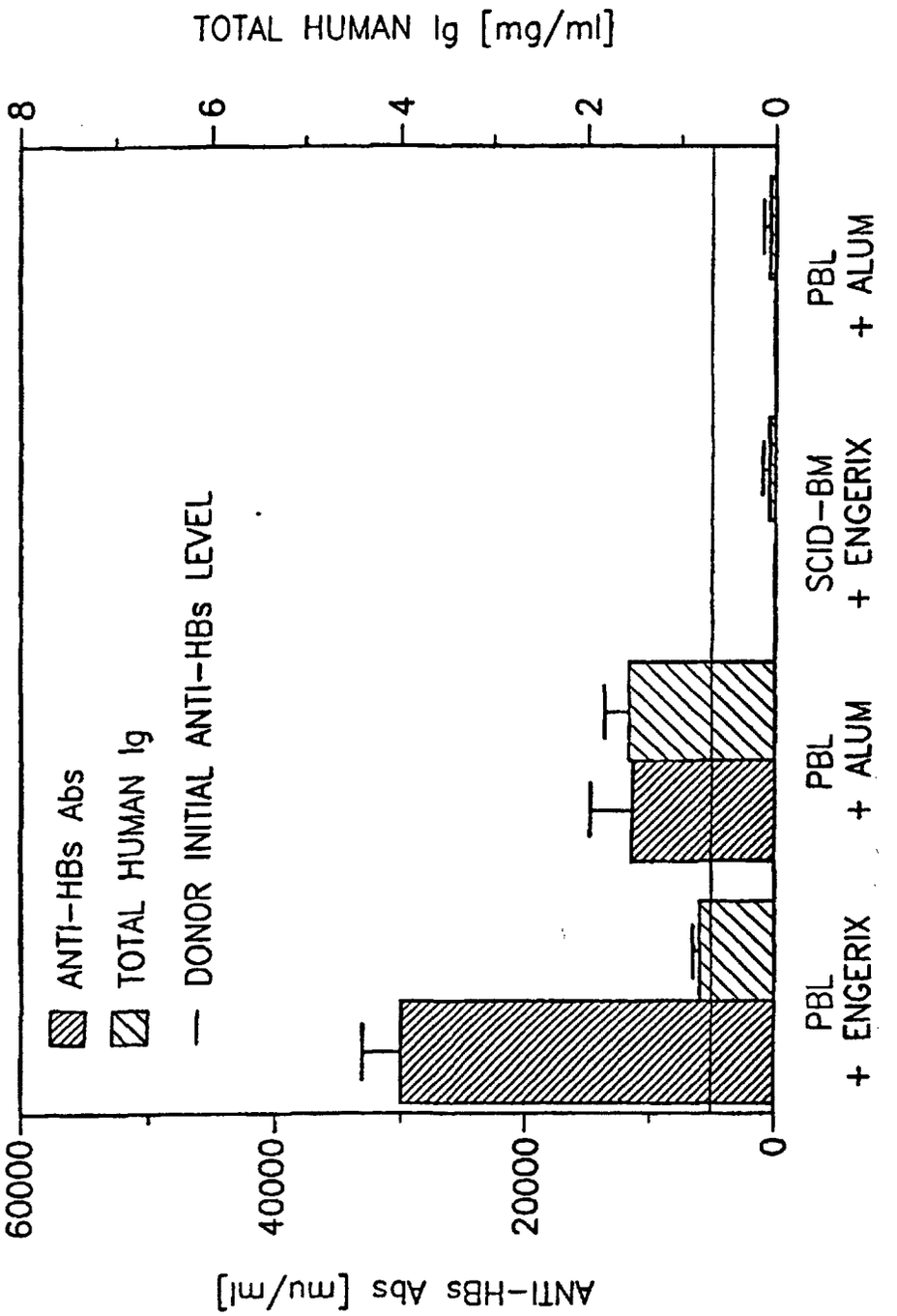
TOTAL HUMAN Ig [mg/ml]
8
6
4
2
0
ANTI-HBs Abs [mu/ml]
60000
40000
20000
0
ANTI-HBs Abs
TOTAL HUMAN Ig
DONOR INITIAL ANTI-HBs LEVEL
PBL + ENGERIX
PBL + ALUM
SCID-BM + ENGERIX
PBL + ALUM

FIG. 1

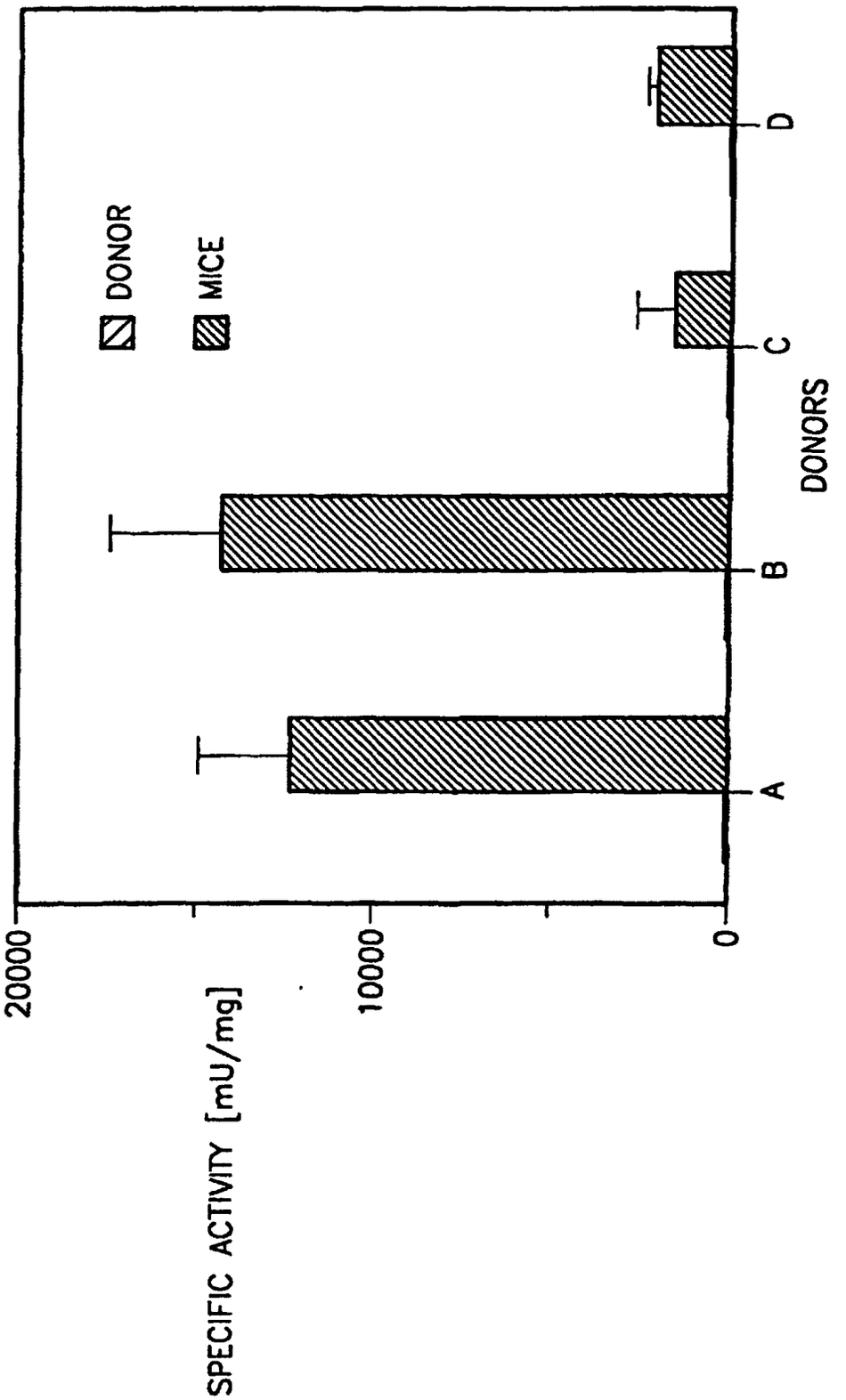
DONOR
MICE
SPECIFIC ACTIVITY [mU/mg]
20000
10000
0
A
B
C
D
DONORS

FIG.2

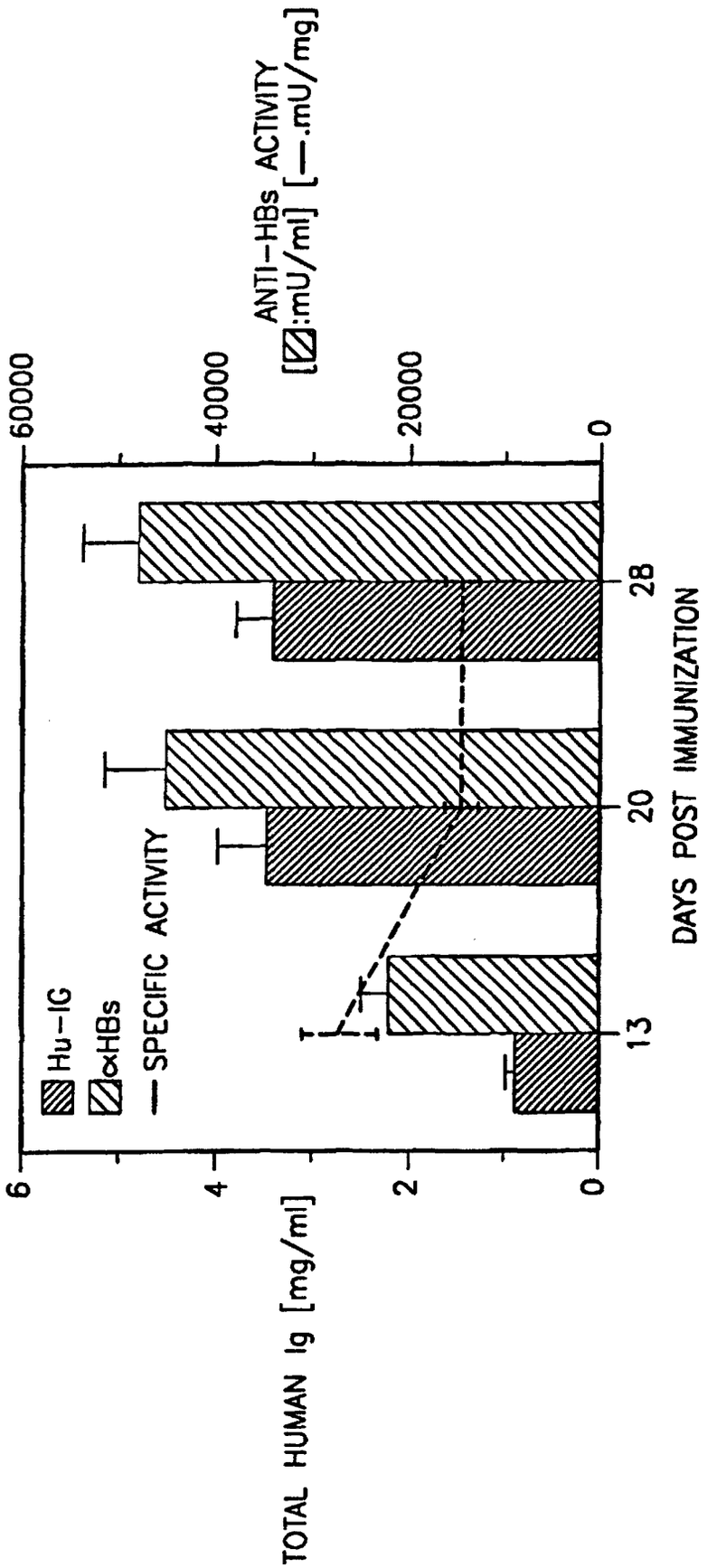
ANTI-HBs ACTIVITY
[▨:mU/ml] [—.mU/mg]
60000
40000
20000
0
28
20
13
DAYS POST IMMUNIZATION
Hu-IG
αHBs
SPECIFIC ACTIVITY
6
4
2
0
TOTAL HUMAN Ig [mg/ml]

FIG.3

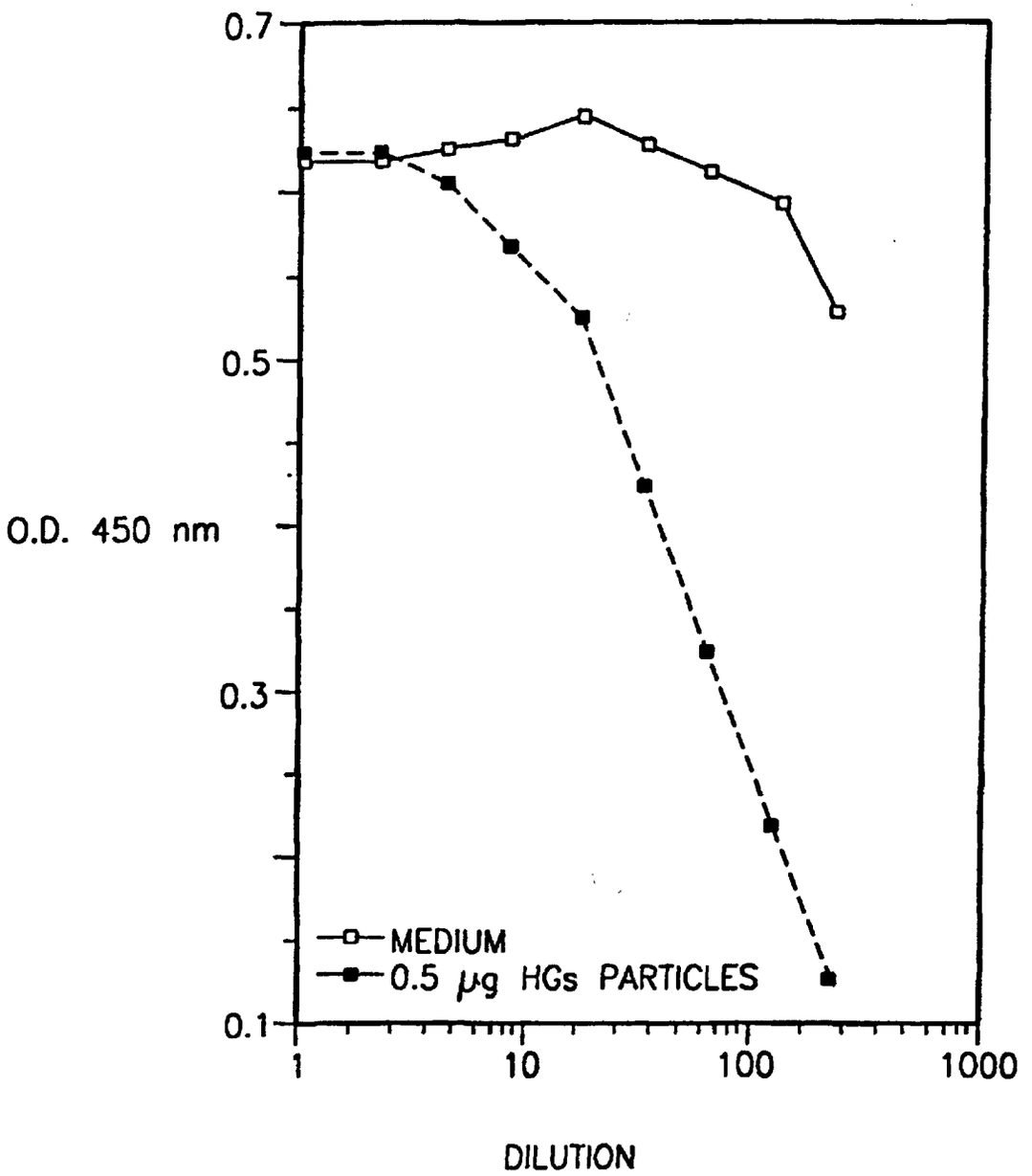
0.7
0.5
0.3
0.1
O.D. 450 nm
MEDIUM
0.5 μg HGs PARTICLES
1
10
100
1000
DILUTION

FIG.4

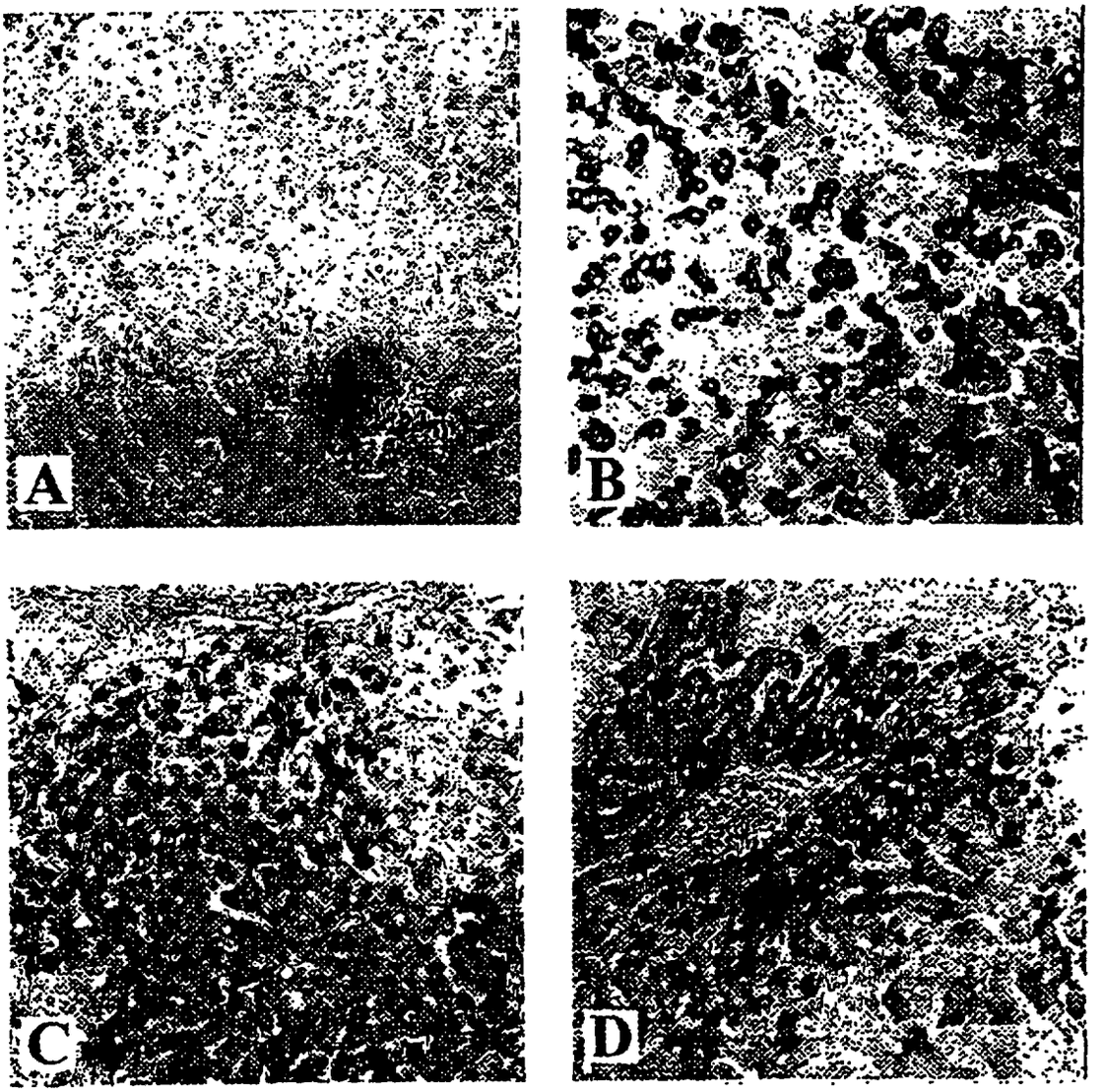

FIG.5

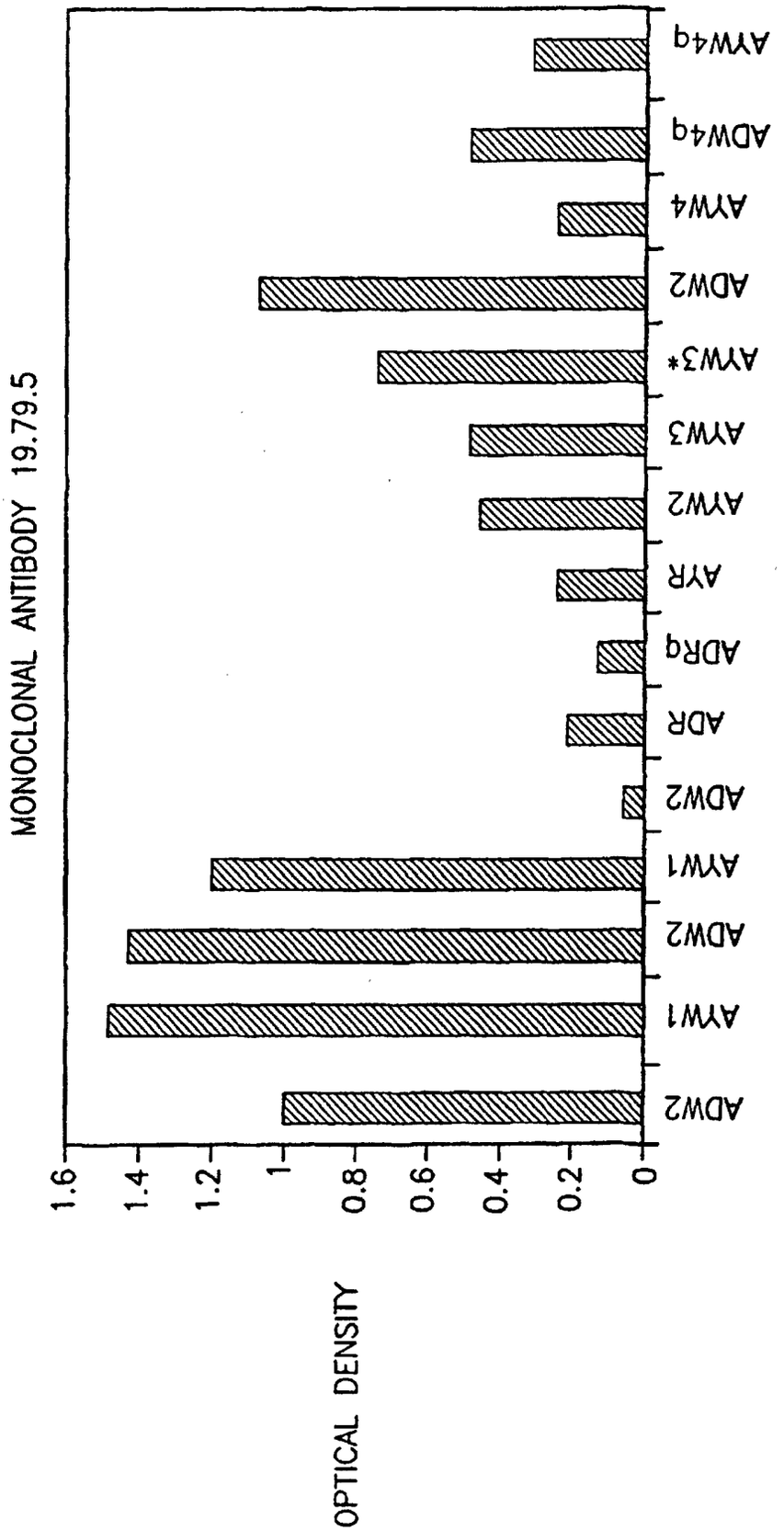
MONOCLONAL ANTIBODY 19.79.5
OPTICAL DENSITY
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ADW2
AYW1
ADW2
AYW1
ADW2
ADR
ADRq
AYR
AYW2
AYW3
AYW3*
ADW2
AYW4
ADW4q
AYW4q

FIG.6

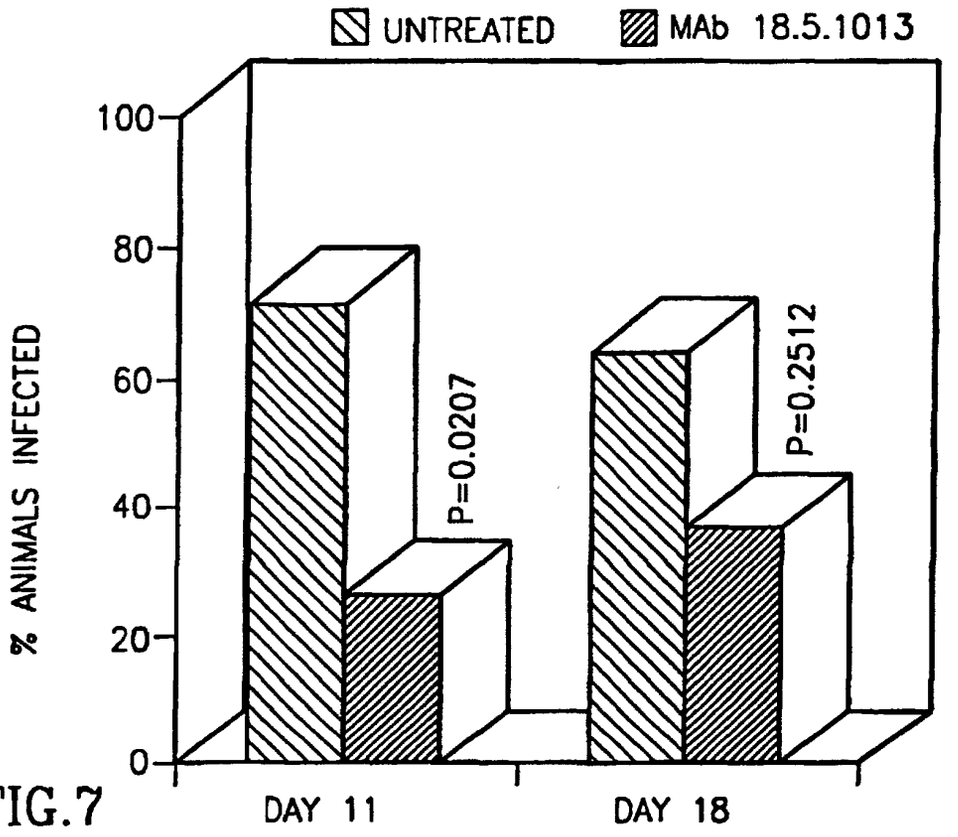
UNTREATED
MAb 18.5.1013
% ANIMALS INFECTED
100
80
60
40
20
0
P=0.0207
P=0.2512
DAY 11
DAY 18

FIG.7

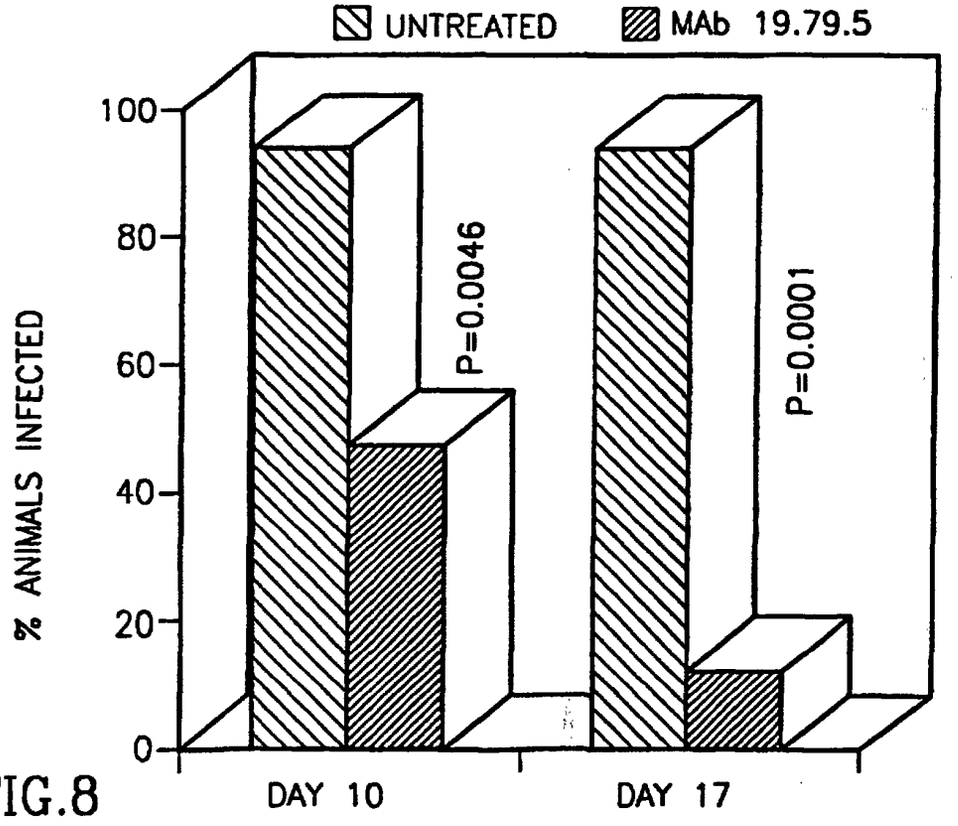
UNTREATED
MAb 19.79.5
% ANIMALS INFECTED
100
80
60
40
20
0
P=0.0046
P=0.0001
DAY 10
DAY 17

FIG.8

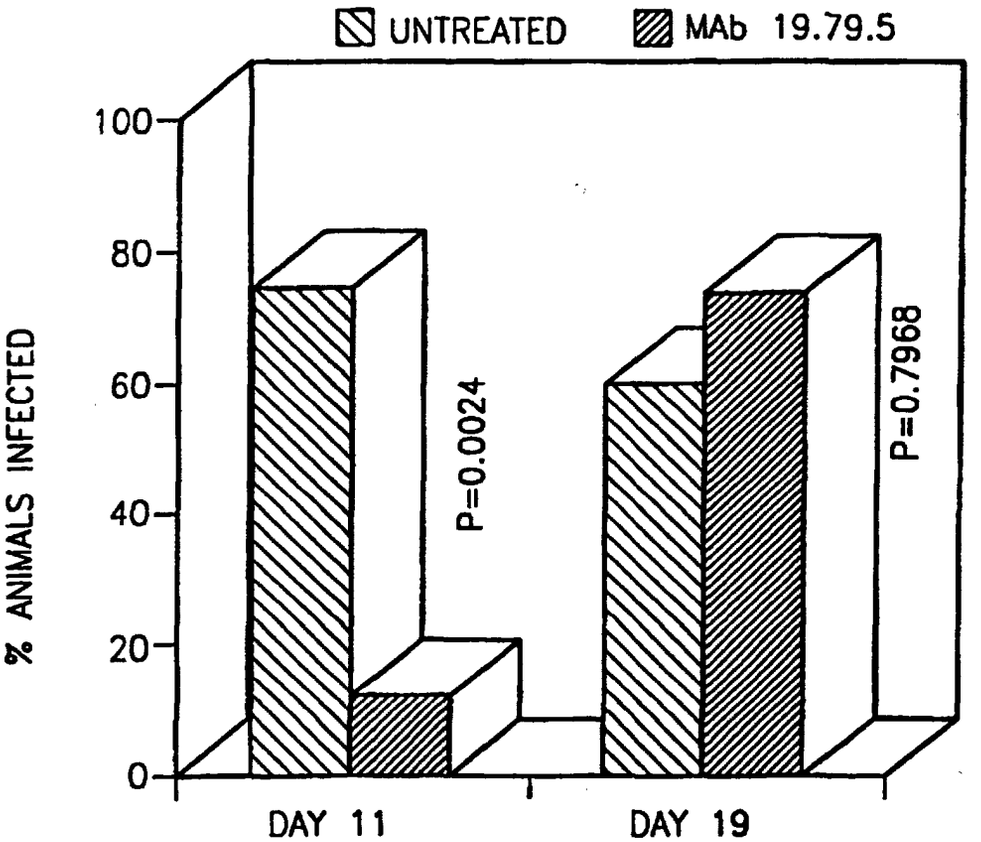
UNTREATED
MAb 19.79.5
% ANIMALS INFECTED
100
80
60
40
20
0
P=0.0024
P=0.7968
DAY 11
DAY 19

FIG.9

		Ser TCC	Tyr TAT	Val GTG 9	Leu CTG	Thr ACT	Gln CAG 18	Pro CCA	Pro CCC	Ser TCG 27	Val GTG	Ser TCA	Val GTG 36	Ala GCC	Pro CCA	Gly GGA 45	Lys AAG	Thr ACG	Ala GCC 54
Arg AGG	Ile ATT	Ser TCC 63	Cys TGT	Gly GGG	Gly GGA 72	Asn AAC	Asn AAC	Ile ATT 81	Gly GGA	Thr ACT	Lys AAA 90	Asn AAT	Val GTG	His CAC 99	Trp TGG	Tyr TAC	Gln CAG 108	Gln CAG	Lys AAG
Pro CCA	Gly GGC	Gln CAG 123	Ala GCC	Pro CCT	Val GTG 132	Leu CTG	Val GTC	Val GTC 141	Tyr TAT	Ala GCT	Asp GAT 150	Ser AGC	Asp GAC	Arg CGG 159	Pro CCC	Ser TCA	Gly GGG 168	Ile ATC	Pro CCT
Glu GAG	Arg CGA	Phe TTC 183	Ser TCT	Gly GGC	Ser TCC 192	Asn AAC	Ser TCT	Gly GGG 201	Asn AAC	Thr ACG	Ala GCC 210	Thr ACC	Leu CTG	Thr ACC 219	Ile ATC	Ser AGC	Arg AGG 228	Val GTC	Glu GAA
Val GTC	Gly GGG	Asp GAT 243	Glu GAG	Ala GCC	Asp GAC 252	Tyr TAT	Tyr TAC	Cys TGT 261	Gln CAG	Val GTG	Trp TGG 270	Asp GAT	Ser AGT	Val GTT 279	Ser AGT	Tyr TAT	His CAT 288	Val GTG	Val GTA
Phe TTT	Gly GGC	Gly GGA 303	Gly GGG	Thr ACC	Thr ACG 312	Leu CTG	Thr ACC	Val GTC 321	Leu CTA	Gly GGT									

FIG.10

EP 0 912 611 B9 (W1B1)

		Gln CAG	Val GTG	Gln CAG 9	Leu CTG	Val GTG	Glu GAG 18	Ser TCT	Gly GGG	Gly GGA 27	Gly GGC	Val GTG	Val GTC 36	Gln CAG	Pro CCT	Gly GGG 45	Gly GGG	Ser TCC	Leu CTG 54
Arg AGA	Leu CTC	Ser TCC 63	Cys TGT	Ala GCA	Pro CCG 72	Ser TCT	Gly GGA	Phe TTC 81	Val GTC	Phe TTC	Arg AGG 90	Ser AGT	Tyr TAT	Gly GGC 99	Met ATG	His CAC	Trp TGG 108	Val GTC	Arg CGC
Gln CAG	Thr ACT	Pro CCA 123	Gly GGC	Lys AAG	Gly GGG 132	Leu CTT	Glu GAG	Trp TGG 141	Val GTG	Ser TCA	Leu CTT 150	Ile ATA	Trp TGG	His CAT 159	Asp GAT	Gly GGA	Ser AGT 168	Asn AAT	Arg AGA
Phe TTC	Tyr TAT	Ala GCA 183	Asp GAC	Ser TCC	Val GTG 192	Lys AAG	Gly GGC	Arg CGA 201	Phe TTC	Thr ACC	Ile ATC 210	Ser TCC	Arg AGA	Asp GAC 219	Asn AAT	Ser TCC	Lys AAG 228	Asn AAC	Thr ACA
Leu TTG	Tyr TAT	Leu TTG 243	Gln CAA	Met ATG	Asn AAC 252	Ser AGC	Leu CTG	Arg AGA 261	Ala GCC	Glu GAA	Asp GAC 270	Thr ACG	Ala GCT	Met ATG 279	Tyr TAC	Phe TTC	Cys TGT 288	Ala GCG	Arg AGA
Glu GAG	Arg AGG	Leu CTG 303	Ile ATT	Ala GCA	Ala GCA 312	Pro CCT	Ala GCT	Ala GCC 321	Phe TTT	Asp GAC	Leu CTC 330	Trp TGG	Gly GGC	Gln CAG 339	Gly GGA	Thr ACC	Leu CTG 348	Val GTC	Thr ACC
Val GTC	Ser TCC	Ser TCG 363																	

FIG.11

EP 0 912 611 B9 (W1B1)