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(54) Title: METHOD OF TREATING HIV INFECTIONS USING IMMUNOTOXINS

(57) Abstract

Disclosed herein are immunotoxins for treating HIV-infected individuals, pharmaceutical formulations comprising these immunotoxins and methods of use thereof. The immunotoxins are conjugates of human monoclonal antibodies directed against HIV proteins (gp41 and p24) and the A chain of the plant toxin ricin.

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METHOD OF TREATING HIV INFECTIONS USING IMMUNOTOXINS

BACKGROUND OF THE INVENTION

The United States Government has rights to this invention by virtue of funding from grants CA-29143, CA-41061, AL-72633 and AI-72658 from the National Institutes of Health.

The Human Immunodeficiency Virus (HIV) has been implicated as the causative agent of Acquired Immune Deficiency Syndrome (AIDS). Two different serotypes of the virus have been identified to date: HIV-1 and HIV-2. It is currently believed that the majority of individuals that become infected with HIV eventually will develop AIDS and are likely to succumb to fatal infections and/or malignancies. At this time, it is estimated that approximately 1.5 million individuals have been infected by HIV in the United States alone.

Several avenues have been explored to treat individuals afflicted with AIDS or having HIV infections. The antiviral drug azidothymidine (AZT) has been found to produce both clinical and immunological improvements upon short-term administration to patients afflicted with AIDS and ARC (AIDS Related Complex - a prodrome of the disease) and to decrease the mortality rate and frequency of opportunistic infections. Although clinical benefits are achieved with AZT, it is costly. A further drawback is that significant drug toxicity often accompanies administration of AZT. This may necessitate blood transfusions and/or reduction of the AZT dosage, or in some instances, discontinuance of AZT therapy altogether. Nonetheless, AZT is the only drug currently authorized for the treatment of AIDS.

An alternative treatment that is currently under evaluation involves administration of one or more lymphokines. Interferon (particularly gamma-interferon), and interleukin-2 are currently being studied for possible use in the treatment of HIV infections. However, the preliminary results of early clinical trials are not promising. Patients receiving lymphokine therapy often suffer serious side effects including low blood pressure, nausea and diarrhea.

It has been proposed to use monoclonal antibodies of defined specificities directed against HIV proteins expressed in infected individuals as therapeutic agents. These virus-encoded proteins are part of the virus particles and are expressed by HIV infected cells and are designated interalia as p24 and gp41. The identification and isolation of gp41 is described in U.S. Patent No. 4,725,669 of M. Essex, issued February 16, 1988 as is its use in the treatment and diagnosis of AIDS. The identification of p24 has been described in Allan, J.S. et al., Science 228: 1091, 1985. However, the use of monoclonal antibodies for the treatment of HIV infections has been hampered because only a limited number of murine and rat monoclonal antibodies to HIV proteins are available. In addition, none of the currently available monoclonal antibodies directed against HIV proteins are of human origin. Administration of murine antibodies to humans can cause dangerous life threatening immunologic reactions, and such antibodies may not be effective in binding to the target HIV proteins in humans.

Stable human cell lines which produce monoclonal antibodies directed against HIV would be useful for treating and/or diagnosing individuals infected with the virus. However, human monoclonal antibodies and particularly those directed against HIV have proven to be far more difficult to produce than those of either rat or mouse origin. Amongst the explanations for this problem are: (a) the most available source of lymphocytes from humans, the peripheral blood, normally contains few antibody producing cells and in some instances, none at all; (b) transformation of antibody producing cells can be achieved using Epstein-Barr virus (EBV), but

the level of antibody production by these transformed cells is often low and unstable; (c) stability of antibody production can be enhanced, as can levels of antibody production, by fusion of EBV-transformed lines to mouse myeloma cells but, these 'heterohybridomas' readily delete human chromosomes and immunoglobulin production is often lost; and (d) fusion of normal or transformed B cells to human lymphoblastoid lines or to heteromyelomas stabilizes antibody production but, until recently, few satisfactory parent lines of this cell type have been available.

Immunotoxins are conjugates of antibodies and either holotoxins or their active A chains (see below) which can be used as cell specific cytoxic agents. Toxins are products of nature usually produced by bacteria and plants. The antibody portion of immunotoxins is used to specifically target the toxin thereby creating cell-specific cytotoxic agents. Immunotoxins comprising specific human antibodies directed against HIV proteins could potentially provide effective treatments for individuals suffering from infections with HIV. However, as mentioned above, human antibodies directed against HIV are not readily available.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide therapeutic agents for treating individuals infected with HIV and having a low non-specific toxicity.

Another object of the present invention is to provide methods employing immunotoxins to treat a mammal suffering from HIV infections.

A further object of the present invention is to provide pharmaceutical formulations comprising effective amounts of immunotoxins for treating mammals suffering from HIV infections.

These and other objects of the present invention will be apparent to those of ordinary skill in the art in light of the present specification.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a radio-immunoprecipitation assay of [^{125}I]-

labelled HIV lysate with serum from an HIV-infected subject or with human monoclonal antibodies from a subset of the lymphoblastoid cell lines used in the present invention.

Fig. 2 is a Western Blot analysis of a subset of the human monoclonal antibodies used in the present invention.

Fig. 3(a-g) are graphs of the growth kinetics and immunoglobulin production of a subset of the human lymphoblastoid cell lines of the present invention.

Fig. 4 is a graph showing the inhibition testing of human monoclonal antibodies 120-16 and 71-31.

Fig. 5 is an SDS-PAGE analysis of two of the immunotoxins of the present invention.

Fig. 6 is a graph showing the inhibition of protein synthesis in HIV-infected and uninfected H9 and U937 cells treated with two of the immunotoxins of the present invention.

SUMMARY OF THE INVENTION

The present inventors have discovered new agents for treating human immunodeficiency virus infections. These agents are human monoclonal antibodies or their fragments directed against HIV proteins gp41 and p24 covalently coupled to one or more toxins, drugs, radionuclides or cytotoxic agents, the conjugates being alternatively referred to herein as immunotoxins.

In one aspect, the present invention comprises an immunotoxin comprising a conjugate of a human monoclonal antibody directed against human immunodeficiency virus gp41 and the A chain of ricin.

In another aspect, the present invention comprises a conjugate of a human monoclonal antibody directed against human immunodeficiency virus p24 and the A chain of ricin.

A further aspect of the present invention comprises a pharmaceutical formulation for treating a mammal suffering from an infection caused by HIV comprising an effective amount of an immunotoxin comprising a human monoclonal antibody directed against human immunodeficiency virus gp41 and the A chain of ricin.

A still further aspect of the present invention

comprises a method for treating a mammal suffering from an infection caused by HIV comprising administering to a mammal in need of such treatment an effective amount of an immunotoxin comprising a conjugate of human monoclonal antibody directed against human immunodeficiency virus gp41 and the A chain of ricin.

DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications and literature references cited in this specification are hereby incorporated by reference in their entirety.

The present inventors have discovered novel therapeutic agents for treating individuals suffering from infections caused by HIV. These are human monoclonal antibodies directed against HIV proteins p24 and gp41 covalently coupled to one or more toxins, drugs, radionuclides, or cytotoxic agents, the conjugates hereinafter alternatively referred to as immunotoxins. The monoclonal antibodies target the cytotoxic agents specifically to HIV-infected cells which express proteins encoded by the virus. The immunotoxins can thus selectively kill HIV-infected cells and may help to curtail or palliate the disease.

The immunotoxins of the present invention can be utilized for the treatment of mammals suffering from HIV infections or AIDS. Due to their potency and lack of non-specific cellular toxicity, the immunotoxins of the present invention may be particularly useful as specific anti-HIV therapeutic agents. Currently there are no agents available to treat HIV infections without concurrent cytotoxicity.

The major HIV-encoded proteins produced by infected cells include gp120, gp41 and p24. Although gp120 shows significant heterogeneity in structure and antigenicity amongst different isolates of HIV, the gp41 protein is relatively conserved, perhaps because it is responsible for anchoring the viral protein gp120 to the plasma membrane of infected cells. Because gp41 is relatively conserved, monoclonal antibodies or their fragments specific for gp41 are employed in one embodiment to construct the immunotoxins of the present

invention. In an alternative embodiment, monoclonal antibodies directed against HIV protein p24 are employed to construct the immunotoxins. The use of p24 antibodies is preferred because this virally-encoded protein is highly conserved in different clinical isolates of HIV-1 and HIV-2.

Immunotoxins generally consist of cell-reactive monoclonal antibodies covalently coupled to toxins, their catalytic subunits or ribosome-inactivating proteins. Toxins, like antibiotics, are products of nature produced by bacteria and plants. The toxins produced by plants show conservation in structure and function. All of the known plant toxins which inhibit protein synthesis are heterodimers (consisting of an A and a B chain) containing disulfide bonds. The B chain is involved in binding of the toxin to the cell surface and is usually a galactose specific molecule of approximately 30 kilodaltons (kd). The A chain is the toxic moiety and is an enzyme which inhibits protein synthesis in eukaryotic cells by modifying one or two nucleoside residues of 28S ribosomal RNA present in the ribosomal 60S subunits.

Ricin is a toxin produced by the beans of the plant Ricinus communis. Both chains of ricin (A and B) have oligosaccharide moieties having a high content of mannose and the A chain contains fucose. After ricin binds to the cell surface, the complex of toxin and its cellular receptor to which it is bound is endocytosed (taken up into endosomes), the disulfide bond between the A and B chain is reduced, and the A chain translocates across an endocytic membrane and gains access to the cytoplasm. The A chain then has access to the 60S ribosomal subunits where it can inhibit protein synthesis and exert its cell killing effect. Immunotoxins containing the A chain of ricin have been utilized to specifically kill neoplastic cells in vivo and in vitro (Vitetta, E. et al., Science 238: 1098-1104, 1987; Blakey, D.C. Adv. Allergy in press; Pastan, I. et al. Cell 47: 642-648, 1986). Ricin A chains are preferred as the toxic moiety of the immunotoxins of the present invention because ricin A chains can be obtained in a pure form (free of B chain) thereby removing non-specific

toxicity. In addition, immunotoxins made with ricin A chains are extremely potent, very specific and have been demonstrated to be safe and effective when administered to patients (for non-HIV induced diseases).

In a preferred embodiment of the present invention, the A chain of ricin is employed as the toxic moiety of the immunotoxin. Pure A chains retain their full ribosome-inactivating function and are non-toxic to cells in vitro at concentrations that are 10^5- to 10^6-fold higher than ricin itself. This is due to the fact that the A chain cannot bind to cells effectively without a B chain. The deglycosylated A chain of ricin (dgA) is particularly preferred since this procedure prevents the Kupffer cells of the liver from rapidly removing the immunotoxin from the circulation after administration to patients. Deglycosylation prevents the immunotoxin from binding to these liver cells which express mannose receptors and thus rapidly remove the immunotoxin-containing mannosylated ricin A chains from the circulation and prevent the immunotoxin from reaching the target tissue.

Deglycosylation of ricin A chains can be performed as described in Example 7 below. Alternatively, as ricin A chains have been cloned in E. coli (O'Hare, M. et al., FEBS Letters 216: 73, 1986) and the proteins produced by bacteria are not glycosylated, deglycosylated A chains can be obtained by expressing the recombinant A chain in bacteria and purifying it from bacterial extracts using techniques well-known in the art.

Ricin A chains for use in the present invention are commercially available from Island Laboratories (Austin, TX) or can be obtained by extraction of the holoprotein and purification from African castor beans (Croda Premier Oils, Hull, England) and by purification of the A chains as described in Fulton, R.J. et al. (J. Biol. Chem. 261: 5314-5319, 1986).

As mentioned above, immunotoxins containing native or deglycosylated ricin A chains are coupled to cell-reactive antibodies by cross-linkers which contain a disulfide bond. The disulfide bond between the antibody and toxin must be cleaved before the A chain translocates into the cytoplasm.

Therefore, the immunotoxins of the present invention comprise a disulfide group in a linker, such as 4-succimidyloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene (SMPT). In a preferred embodiment of the present invention, the immunotoxin comprises purified human monoclonal anti-gp41 or its Fab' fragment (see below) coupled via one of two heterobifunctional thiol-containing linkers SMPT to the deglycosylated A chain of ricin. SMPT is particularly preferred because it is the most stable in vivo (Thorpe et al., Cancer Res. 48: 693, 1988). Examples of other linkers which could be used with the monoclonal antibodies of the present invention to produce the immunotoxins include N-succinimidyl-3-(2-pyridyldithio-propionate (SPDP) and N-succinimidyl-5-thioacetate (SATA). SPDP is commercially available from numerous commercial sources such as Pharmacia Fine Chemicals, Piscataway, NJ, Pierce Chemical Co., Rockford, IL and Sigma Chemical Co., St. Louis, MO. SMPT can be synthesized and used as described in Thorpe et al. (Cancer Res. 47: 5924-5931, 1987). SATA is commercially available from Calbiochem, La Jolla, CA. The use of SATA is described in Duncan et al., Anal. Biochem. 132: 68-73, 1983.

Fab' fragments are partial proteolytic digests of intact antibodies and contain the antigen binding sites of the antibody molecule. The preparation and use of Fab' fragments in immunotoxins is described in Ghetie, M-A et al. Cancer Res. 48: 2610-2617, 1988.

As shown in Example 8 below, the immunotoxins of the present invention were found to selectively reduce protein synthesis in HIV-infected T-cells (H9 cells) while having no toxicity for uninfected, control cells at concentrations 3 orders of magnitude higher. Over 1,000 patients have been treated with ricin A chain-containing immunotoxins for various malignancies and the immunotoxins have been found to be safe and effective.

In addition, the immunotoxins of the present invention also showed selective toxicity for HIV-infected monocytes (U937 cells) as shown in Example 8 below. This is a most important finding in that there is accumulating evidence that monocytes

may be reservoirs for the virus in infected patients and effective treatment modalities for HIV-infected individuals should be directed towards these infected cells as well as T-cells.

Non-limiting examples of other toxins which may be used with the monoclonal antibodies of the present invention to produce immunotoxins include diphtheria toxin, arbin and/or ribosome inactivating proteins (RIPs) such as gelonin (Blakey et al. Adv. Allergy, in press; Vitetta, E. et al. Science 238: 1098-1104, 1987).

As shown in Table IV of Example 8 below, the addition of a 5-fold excess of unconjugated anti-gp41 antibody to the anti-gp41 : deglycosylated A chains (dgA) only partially inhibited the immunotoxin-induced cytotoxicity, suggesting that the presence of anti-gp41 antibody in the serum of AIDS patients may not interfere with the specific toxic effects of the anti-gp41 immunotoxins.

The present inventors have also found that the addition of chloroquine ($C_{18}H_{26}ClN_3$, mol. wt. 319.89; Merck Index, Winholz et al. eds., pgs. 303-304, 1983) potentiated the killing of HIV-infected U937 cells by the immunotoxins of the present invention. Chloroquine is known to potentiate the specific killing of cells by immunotoxins possibly because it increases the pH of the lysosomal compartment and inhibits the fusion of endosomes (low pH intracellular vesicles) with lysosomes. By elevating the pH and inhibiting the fusion of endosomes and lysosomes, these substances inhibit the proteolytic digestion of the toxin before it can reach the ribosomes. Therefore, the present invention also comprises administration, either together with or relatively soon after, of an immunotoxin potentiator with the immunotoxins of the present invention in order to treat HIV-infected individuals.

"Immunotoxin potentiator" is defined herein as substances which increase the toxicity of an immunotoxin preferably 2- to 10-fold. When administered to mammals with the immunotoxins of the present invention, effective amounts of chloroquine will range between about 10^{-8}M and 10^{-6}M. The

effective amounts of monensin and NH_4Cl may be determined by routine experimentation using, e.g. the in vitro system described in Examples 7 and 8 below.

Other similar immunotoxin potentiators for use in the present invention include NH_4Cl and monensin (Merck Index, Windholz, M. et al., eds. p.893, 1983). Monensin ($C_{36}H_{62}O_{11}$, mol. wt. 670.9) is the major factor in a complex isolated from Streptomycetes cinnamonensis and its production is described in U.S. Patent No. 3,501,568 issued March, 1970. Chloroquine is commercially available from Sigma Chemical Co., St. Louis, MO and its synthesis has been described in U.S. Patent No. 2,233,970.

The present inventors have isolated eleven human lymphoblastoid cell lines producing human monoclonal antibodies which may be used as the antibody component of the immunotoxins of the present invention. These lymphoblastoid cell lines were formed by immortalizing lymphocytes obtained from HIV seropositive patients by infecting these lymphocytes with Epstein Barr Virus (EBV) in vitro. Blood was obtained from 94 HIV seropositive individuals in two series of immortalizations (see Examples 1-6 below). Peripheral blood mononuclear cells were obtained and incubated overnight with EBV. The infected cells were cultured at 80,000 cells per well in microtiter wells for 3-4 weeks and assayed for anti-HIV antibody production using a non-commercial enzyme linked immunosorbant assay (ELISA, see below) and a commercial ELISA employing HIV-coated beads. The specificity of each positive reaction obtained from the ELISA was confirmed by testing for their non-reactivity on identical beads coated with bovine serum albumin (BSA).

Approximately 9% of the lymphoblastoid cell cultures tested positively in the non-commercial ELISA. For expansion the positive wells were cultured for two more weeks. It was found that 2.4% tested positively for HIV proteins by ELISA and 0.67% proved to be specific for HIV by virtue of their non-reactivity with the BSA beads. The anti-HIV antibodies produced were found to be directed against gp41 and p24 and had sufficient activity to show reactivity by ELISA, Western blot,

radioimmunoprecipitation and/or immunofluorescence. The stable clones were subcultured at doubling dilution on feeder cells and then subcultured 1 to 3 times at 10 or 100 cells per well with irradiated human lymphoblastoid feeder cells and expanded into tissue culture flasks.

The present inventors have performed some epitope mapping of the human monoclonal antibodies used in the present invention. For example, it can be seen from the data presented in Example 5, Table III below that monoclonal antibodies 50-69 98-43 and 126-6 bind to the same epitope region (i.e. amino acids falling between residues 567 and 649) whereas 98-6 binds to a different region (amino acids falling between residues 642 and 692). However, monoclonal antibodies 50-69, 98-43 and 126-6 differ in their epitope specificity as demonstrated by the fact that 50-69 binds to peptide 599-613 whereas 98-43 and 126-6 do not. Three of the anti-p24 antibodies have been tested (i.e. 71-31, 91-5 and 91-6). All bind to the same region of p24 (131-198).

The human monoclonal antibodies used in the present invention are all of the IgG isotype and may be recovered from the supernatants of monoclonal antibody producing lymphoblastoid cell cultures and purified by conventional methods known in the art for the purification of IgG. Such methods include Protein-A Sepharose chromatography, a combination of Affi-gel Blue (Bio-Rad, Richmond, CA) and Protein-A Sepharose chromatography, or High Performance Liquid Chromatography (HPLC).

The eleven stable lymphoblastoid cell lines described in Examples 1-6 below produce human monoclonal antibodies which are directed against unique epitopes which are expressed in HIV-infected patients. Although some epitope mapping has been performed (see Table III in Example 5 below), further epitope mapping will determine the exact specificity of each of the monoclonal antibodies.

The human monoclonal antibodies used in the present invention are directed against either immunodominant (common) or non-dominant epitopes of the gp41 and p24 viral proteins. As employed in this specification, the term "immunodominant"

refers to an antigenic determinant against which essentially all patients respond by producing antibodies against such determinants. Antibodies 50-69 and 120-16, directed against gp41, are to immunodominant epitopes. These two antibodies may be employed for passive immunizations and/or diagnostic reagents. Antibodies 71-31 and 91-5, directed against p24, are to non-dominant epitopes.

The classification of the antibodies used in the invention into an immunodominant or non-dominant grouping was accomplished by inhibition testing as described in Example 6 below. In this assay, sera collected from HIV seropositive individuals were used to inhibit the binding of biotinylated monoclonal antibodies directed against HIV proteins to their respective antigens.

The production and use of human monoclonal antibodies as therapeutic anti-HIV agents is described in a copending U.S. patent application of Susan Zolla-Pazner and Miroslaw K. Gorny filed February 28, 1989 entitled Human Monoclonal Antibodies to Human Immunodeficiency Virus. The production and characterization of the eleven human monoclonal antibodies used to construct the immunotoxins of the present invention is shown in Examples 1-6 below.

Human lymphoblastoid cell lines 91-5 and 126-6 producing monoclonal antibodies directed against HIV proteins p24 and gp41 respectively, have been deposited with the American Type Culture Collection (ATCC, Rockville, MD) and have received accession numbers CRL 10038 and 10037, respectively.

When employed to treat individuals infected by HIV or suffering from AIDS, the immunotoxins of the present invention may be administered to mammals in effective amounts broadly ranging between about 1 mg per kg body weight of said mammals and about 5 mg per kg body weight of said mammals. The immunotoxins of the present invention may also be administered (either with or relatively shortly after the immunotoxins) with an immunotoxin potentiator. Such substances may be added in effective amounts broadly ranging between about 10^{-8}M and about 10^{-6}M.

The immunotoxins of the invention may be administered parenterally, either via the intravenous or intramuscular route. A typical treatment regimen would comprise administration of an effective amount of immunotoxin over between about one week and about 6 months. The number of treatments required to control a patient's disease may vary from individual to individual, depending upon the severity and stage of the illness and the individual characteristics of each patient being treated. The total dose required for each treatment may be administered by multiple doses or in a single dose. The immunotoxins may be administered alone or in conjunction with other HIV treatments, such as AZT, in order to control a patient's disease. The anti-HIV treatment may be administered one or two times a week or more as determined by the patient's condition and the stage of the patient's disease.

The immunotoxins of the present invention can be incorporated into conventional pharmaceutical formulations for use in treating individuals that are afflicted with HIV. The pharmaceutical formulations of the invention comprising an anti-HIV effective amount of the immunotoxins of the present invention with or without an effective amount of the immunotoxin potentiators mentioned above. The quantity of effective dose applied by each injection is relatively unimportant since the total dosage can be reached by administration of one or a plurality of injections. In addition, such formulations may comprise pharmaceutically-acceptable carriers, diluents, salts and other materials well-known in the art. Isotonic saline, sterile water, 10% maltose, human serum albumin, glycine or other pharmaceutically-acceptable materials may be used as diluents, carriers or solvents in preparing the pharmaceutical formulations comprising the immunotoxins of the present invention.

The present invention is described below in specific working examples which are intended to illustrate the invention without limiting its scope.

EXAMPLE 1: IMMORTALIZATION OF HUMAN B-CELLS

Blood was obtained from 58 HIV-seropositive individuals

who were intravenous drug users or homosexuals. The presence of antibody to HIV in the blood was determined using a commercial enzyme-linked immunosorbent assay (ELISA) (Organon-Teknika Bio-Enzabead HTLV-III ELISA, Durham, NC) and confirmed by Western blot (Novapath Immunoblot Assay, Bio-Rad, Hercules, CA and Biotech/DuPont, DuPont, Wilmington, DE). The disease status of patients was established on the basis of an immunologic staging system as described by Zolla-Pazner et al. (Proc. Nat. Acad. Sci. USA 84: 5404, 1987):

Scale Score	T4/T8 ratio	#T4/mm	#lymphocytes/mm
0	> 1.0	> 500	> 1500
1	< 1.0	> 500	> 1500
2	< 1.0	< 500	> 1500
3	< 1.0	< 500	< 1500

Peripheral blood mononuclear cells collected from the patients were obtained by centrifugation of heparinized blood, diluted 1:1 with RPMI-1640 and centrifuged on Histopaque (Sigma, St. Louis, MO) at 300 x g for 30 minutes. Cells at the medium/Histopaque interface were recovered, washed three times and incubated overnight at a density of 2 x 10^6 cells/ml with the filtered supernatant from the EBV-transformed marmoset cell line B95-8 (Proc. Nat. Acad. Sci. USA 70: 190, 1973, available under Accession Number CRL 1612 from the American Type Culture Collection, ATCC, Rockville, MD). Lymphocytes were then washed once and cultured in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (Hyclone Labs, Logan, UT) 2mM L-glutamine, 100U/ml penicillin, and 100 micrograms/ml streptomycin (complete medium) for four weeks in 96-well plates (Costar, Cambridge, MA) at 80,000 cells per well.

EXAMPLE 2: ISOLATION AND SCREENING OF LYMPHOBLASTOID CELL LINES FOR ANTI-HIV ANTIBODY PRODUCTION

After screening for anti-HIV antibody production by a non-commercial ELISA (see below), positive cultures were expanded into wells of 24-well tissue culture plates (Costar) and cultured for two more weeks. All initial and expanded cultures were fed at weekly intervals with complete medium.

Cultures with supernatants showing specific reactivity to HIV were then subcultured one to two times at doubling dilution on feeder layers of irradiated GK-5 human lymphoblastoid cells (derived from a variant of GM1500; Satoh, J. et al., N. Engl. J. Med. 309: 217, 1983) which had been exposed to 3000 Rads of gamma-radiation. Stable clones were then subcultured one to three times at 10 to 100 cells per well on feeder cells and then expanded into flasks.

Thus, initial cultures of immortalized B-cells (hereinafter referred to as lymphoblastoid cell lines) were established and further characterized as described below.

The screening of the initial cultures in 96-well plates was performed using a non-commercial ELISA. Immulon 2 plates (Dynatech, Chantilly, VI) were coated overnight at 4°C with 4 micrograms/ml of HTLV-III_B lysate (purchased from Electro-Nucleonics, Inc., Silver Spring, MD) diluted in carbonate buffer, pH 9.8. Plates were washed three times with phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBS-Tween). The culture supernatants to be assayed (0.1 ml per well) were then added and incubated for 90 or 120 minutes at 37°C. Subsequently, plates were washed with PBS-Tween and incubated with goat anti-human immunoglobulin conjugated to alkaline phosphatase (Organon Teknika-Cappel, Malvern, PA) for another 90 minutes at 37°C. The plates were washed again with PBS-Tween and the substrate, p-nitrophenyl phosphate in 10% diethanolamine, was added for 30 minutes. The reaction was terminated with 25 microliters of 1N NaOH and the absorbance was read at 405 nm in an automated ELISA reader (MR 600 Microplate Reader, Dynatech).

The specificity of the antibody binding was assessed by testing the supernatants for reactivity against HIV-coated beads (Bio-EnzaBead) and against uncoated beads (obtained from Organon Teknika Cappel) which were then coated with bovine serum albumin (BSA, Sigma Chemical Co.) by incubating the beads in 1.25% BSA diluted in PBS for 1 hour at room temperature. Reactivity with HIV-coated beads, but not with BSA-coated beads, was used as a criterion for specificity. Further

analysis of the specificity of the monoclonal antibodies was then carried out by Western blot using a commercially-available kit (Bio-Rad, Richmond, CA) and by radioimmunoprecipitation (RIP). RIP assays were carried out using the method of Pinter and Honnen (J. Immunol. Methods. 112: 235-241, 1988). Briefly, 30 micrograms of HTLV-III_B lysate (purchased from Organon--Teknika), was labeled with 125[I] using the Bolton-Hunter reagent (New England Nuclear, Boston, MA). Bound label was separated from free label on a Bio-Gel P-4 column (Bio-Rad). Fifty microliters of culture supernatant were incubated with 5 x 10^6 cpm of the labeled lysate for 1 hour at 37°C, then 50 microliters of 10% fixed Staphylococcus aureus (Pansorbin, Calbiochem, La Jolla, CA) was added. The immunoprecipitate was washed three times by centrifugation and the air-dried pellet was resuspended in buffer, boiled for 3 minutes and electrophoresed on a 10% SDS polyacrylamide gel. The gels were dried and exposed for one to three days to X-Omat S film (Kodak, Rochester, NY).

The class and light chain type of anti-HIV antibody was determined by ELISA. For these assays, microtiter plates (Immulon 2) were coated with 4 micrograms/ml of HIV lysate (Electro-Nucleonics) and then incubated with culture supernatants. The type of antibody binding to HIV was determined using the following alkaline phosphatase-coupled antibodies: goat anti-human IgG (gamma specific), goat anti-human kappa chain and goat anti-human lambda chain (Organon Teknika-Cappel, Malvern, PA). The subtype of the monoclonal antibody was also analyzed by ELISA using alkaline phosphatase-labeled mouse monoclonal antibodies against the four subclasses of human IgG (Zymed, San Francisco, CA).

Immunoglobulin quantitation was also performed by ELISA. Immulon 2 plates were coated with goat anti-human IgG (gamma specific) and incubated with culture supernatants. Bound IgG was detected with alkaline phosphatase-labeled goat anti-human IgG (gamma specific). Affinity-purified human IgG (Cappel) was used to produce standard curves.

A total of 14,329 cultures in microtitre wells were es-

tablished using cells derived from the 58 subjects. Approximately half of these cultures were derived from three serial bleeds from a single subject (with a scale score of 1) over a period of three months. The remaining wells were established using cells derived from 57 subjects whose scale scores ranged from 0 to 3. The results of this procedure are shown in Table I below.

TABLE I

QUANTITATIVE RESULTS OF THE PROCEDURE USED TO PRODUCE HUMAN MONOCLONAL ANTIBODIES TO HIV

	No. of wells	% positive wells
1. Infection of PBMC with EBV	14,329	(100)
4 weeks		
Screen for anti-HIV by Non-commercial ELISA	1,290	9.0
2. Expand positive wells		
2 weeks		
Screen for anti-HIV by: Non-commercial ELISA	573	4.0
Commercial ELISA	340	2.4
Screen for specificity (reactive HIV, unreactive with BSA)	97	0.67
Screen for reactivity by RIP	57	0.40
3. Subculture positive wells by doubling dilution (10,000-10 cells/well)		
4-6 weeks		
Screen by commercial ELISA	16*	0.11*
4. Subculture positive wells at (100 and 10 cells/well)		
Screen by commercial ELISA	7*	0.05*

* No. and % of positive plates from subcultures of individual wells which contain at least one antibody positive well.

After four weeks, 9% of the wells displayed antibody production as revealed by the non-commercial ELISA (Table I). After expansion, only 2.4% of the original cultures continued to produce antibody reactive with a commercial ELISA kit and only one-quarter of these (0.67% of the original 14,329 cultures) were producing antibodies which reacted specifically with HIV.

To determine whether the severity of disease in the cell donor affected the number of cultures able to produce antibodies and the specificity of antibody produced, the seropositive cell donors were categorized with respect to disease status using the immunologic staging system of Zolla-Pazner et al. (supra) and the results are shown in Table II below.

TABLE II

CHARACTERISTICS OF CELL CULTURES DERIVED FROM PATIENTS AT DIFFERENT STAGES OF HIV INFECTION

Scale Score	No. of patients	No. of wells	No. of positive wells (Includes HIV-specific & non-specific reactivity)	No. of wells with HIV-specific antibody	No. of clumps per well+
HIV-seronegative patients:					
	3	637	0	0	7.3
HIV-seropositive patients:					
0	4	725	18 (2.4%)	6 (0.8%)	0.92
1	13	8,789	180 (2%)	66 (0.7%)	0.88
2	20	2,792	54 (1.9%)	16 (0.5%)	0.65
3	21	2,023	88 (4.3%)	9 (0.4%)	0.22
	58	14,329	340 2.4%	97 0.67%	0.66

* Specificity of anti-HIV antibodies were assessed by commercial ELISA using HIV-coated and BSA-coated beads.

\+ B cells transformed by EBV stick together and create characteristic clumps of cells which were quantitated microscopically.

The results, shown in Table II, revealed that cultures obtained from patients with a scale score of 3 (severe immunodeficiency) gave a higher percentage of antibody producing wells than patients with lesser scale scores. However, only 10% (9/88) of reactive wells from the cells of stage 3 patients were specifically reactive with HIV whereas 30-37% of wells from cells of patients with scale scores of 0-2 were specifically reactive with HIV. Thus, after six weeks of culture, cells from patients with lower scale scores produced a higher percentage of wells containing HIV-specific antibody.

Analysis of antibodies from ELISA-positive expanded cultures was carried out by RIP. Only 59% of supernatants from these cultures were also positive on RIP analysis. RIP analysis demonstrated that, out of 57 supernatants, 44 showed reactivity to env-encoded proteins, 11 to gag proteins and 2 to reverse transcriptase.

Therefore, specific lymphoblastoid cell lines were isolated and further cloned as described below.

EXAMPLE 3: SPECIFICITY AND REACTIVITY OF THE HUMAN MONOCLONAL ANTIBODIES OF A SUBSET OF THE PRESENT INVENTION

The 58 cell lines mentioned above were then cloned by doubling dilution from 10,000 to 10 cells per well. Wells with the lowest cell concentration which were producing antibodies were then picked and cloned at 100 or 10 cells per well. Using this procedure, seven cell lines, 3 producing anti-gp41 antibodies and 4 producing anti-p24 antibodies were established which have been cloned from one to three times at 100 or 10 cells per well. The reactivities of the antibodies from these lines are shown in Figures 1 and 2.

All seven of the cell lines of this Example produced antibodies of the IgG subtype as shown in Figure 1 and Table III. As shown in Figure 1 below, HIV-positive control sera and 6 out of 7 of the monoclonal antibodies tested reacted with [^{125}I]-labeled HIV lysate. In lane 1, reactivity with serum from an HIV-infected patient by radioimmunoprecipitation (RIP) is shown. In lane 2, reactivity with antibody from cell line 50-69, lane 3, reactivity with antibody from cell line 98-6,

lane 4, reactivity with antibody from cell line 98-43, lane 5, reactivity with antibody from cell line 71-31, lane 6, reactivity with antibody from cell 91-5, lane 7, reactivity with antibody from cell line 91-6 and lane 8, reactivity with antibody from 98-4.9. The molecular weights of the major viral proteins are shown on the left in kilodaltons. Antibody from three of the cell lines bound to env-encoded protein gp41 (lines 50-69, 98-6 and 98-43, lanes 2-4 respectively). Antibodies from four of the cell lines bound to gag encoded protein p24 (lines 71-31, 91-5, 91-6 and 98-4.9, lanes 5-7 respectively). By RIP, antibodies from cell lines 71-31, 91-5 and 91-6 reacted with p24. Antibodies from 98-4.9 were unreactive by RIP since IgG_3, the subtype of this antibody, does not bind to Protein A and is therefore not precipitated. Antibody from all 4 of these anti-gag cell lines were also tested by Western blot (Figure 2) and reactive with gag products.

In Figure 2, Western Blot strips were used to show the reactivity of serum from a normal control (lane 1), an HIV-infected subject (lane 2), and of supernatant from cell lines 71-31 (lane 3), 91-51 (lane 4), 91-6 (lane 5), and 98-4.9 (lane 6). Western blot analysis showed all 4 monoclonal antibodies reacted with p24 and with known gag precursor p55 and intermediate p40. All four antibodies also reacted with a breakdown product of p24 which migrated with a mobility of approximately 22 kilodaltons (kd); three of the four anti-gag monoclonal antibodies also reacted with additional intermediate precursors which were noted with a mouse monoclonal antibody and which had mobility of approximately 37, 31 and 28 kd.

Studies of the growth characteristics and level of antibody production of each line were performed. Each cell line was initially cultured in replicate wells at 0.5×10^6 cells/ml for 1-8 days. The number of cells and the amount of immunoglobulin produced is shown in Figure 3. In Figure 3, at each time point, separate wells were used to test for viable cell number (closed circles) and for secreted human IgG (open circles). Cell lines studied were 50-69(a), 98-6(b), 98-43(c), 71-31(d), 91-5(e), 91-6(f) and 98-4.9(g).

Peak cell density was noted at 4 days with maximum densities of 1.0 to 2.4 x 10^6 cells/ml. The doubling time of cells in log phase ranged from 40-61 hours. The concentration of immunoglobulin produced varied widely, generally peaking at day 5 of culture and ranging from 9-112 micrograms/ml.

It should be noted that cell line 98-4.9 has not been stabilized with respect to the production of human monoclonal antibodies.

EXAMPLE 4: GENERATION OF ADDITIONAL LYMPHOBLASTOID CELL LINES PRODUCING HUMAN MONOCLONAL ANTIBODIES TO HIV

Peripheral blood mononuclear cells were obtained from another 36 HIV-seropositive individuals, the cells immortalized by EBV infection, screened and selected as in Examples 1-3 above. Positive cultures were expanded, subcultured by doubling dilution and again subcultured one to three times at 10 to 100 cells per well. Four stable lymphoblastoid cell lines producing human monoclonal antibodies were obtained as follows: 120-16, 126-6 and 126-50 directed against gp41; and 134-F6 directed against p24.

Each of the monoclonal antibodies of this Example was tested for its specificities by means of commercial ELISA (the supernatants were reactive with HIV-coated beads and unreactive with BSA-coated beads), by radioimmunoprecipitation and by Western Blot as in Example 3 above. The characteristics of these human monoclonal antibodies are shown in Table III below.

EXAMPLE 5: CHARACTERIZATION OF THE HUMAN MONOCLONAL ANTIBODIES OF THE PRESENT INVENTION

The specificity and biological activities of the human monoclonal antibodies of the present invention are summarized in Table III below.

TABLE III

Characteristics of human monoclonal antibodies against HIV

Cell line	Isotype, Subclass and Light Chain Type	RIP	ELISA			Western Blot		Function			Approx. Epitope Region
			ENV9	PE3	p121	r-gp41	r-p24	Neutr.	Enhanc.	ADCC	
50-69	IgG_2k[1]	gp41	+	-	+	+	-	-	-	+	573 - 642[2]
98-6	IgG_2k	gp41	+	-	-	+	-	-	-	+	642 - 692
98-43	IgG_2k	gp41	+	-	±	+	-	-	-	+	573 - 642
120-16	IgG_2k	gp41	+	-	-	NT[3]	-	-	+	+	NT
126-6	IgG_2k	gp41	+	-	+	NT	-	-	-	+	567 - 649
126-50	IgG_2k	gp41	+	-	-	NT	-	-	-	+	NT
71-31	IgG_1l[4]	p24	-	-	-	NT	+	-	-	-	131 - 198[5]
91-5	IgG_1l	p24	-	-	-	NT	+	-	-	-	131 - 198
91-6	IgG_1l	p24	-	-	-	NT	+	-	-	-	131 - 198
98-4.9[6]	IgG_3l	p24	-	-	-	NT	NT	-	-	-	NT
98-4.3	IgG_1l	p24	NT	NT	NT	NT	NT	NT	NT	NT	NT
134-F6	IgG_3k	p24	NT	NT	NT	NT	NT	NT	NT	NT	NT

1 k = Kappa

2 The numbering system according to J. Virol. 61: 570, 1987.

3 NT = Not tested

4 l = Lambda

5 The numbering system according to Wain-Hobson, et al., Cell 40: 9, 1985

6 This lymphoblastoid cell line has not been stabilized with respect to the production of monoclonal antibodies

In Table III, epitope mapping was performed using recombinant antigens using Western Blot and ELISA formats. Viral Neutralization and enhancement assays were performed as in J. Clin. Micro. 26: 231, 1988. ADCC assays were performed according to Lyerly, H.K. et al. AIDS and Human Retroviruses 3: 409-422, 1987. Western Blot analysis was performed using recombinant p24 or gp41 (produced by Organon-Teknica).

ELISA's were performed using cloned gp41 antigens. ENV9 is a cloned gp41 protein encompassing residues 461 to 761 (obtained from DuPont, Wilmington, DE). PE3 is a 286 amino acid sequence from gp120 (obtained from DuPont, Wilmington, DE). p121 contains residues 561-649 of gp41 (obtained from Centocor, Malvern, PA).

As can be seen in Table III above, all of the anti-gp41 human monoclonal antibodies of the present invention were of the IgG isotype and mediated antibody dependent cellular cytotoxicity (ADCC).

In addition, epitope mapping showed that some of the anti-gp41 antibodies bound to different epitopes on the viral protein. Monoclonal antibodies 50-69 and 98-43 bound to a peptide encompassing residues 573-642; monoclonal antibody 98-6 was directed against a peptide encompassing residues 642-692. The numbering system for the gp41 peptide is according to J. Virol. 61: 570, 1987.

All three of the anti-p24 monoclonal antibodies tested (71-31, 91-5 and 91-6) bound to a HIV p24 peptide encompassing residues (131-198 (the numbering system according to Wain-Hobson et al. Cell 40: 9, 1987) (Table III) and were of the IgG isotype.

Finally, none of the monoclonal antibodies of the present invention were able to neutralize the infectivity of HIV and one (120-16) enhanced viral pathogenicity.

EXAMPLE 6: INHIBITION TESTING OF THE MONOCLONAL ANTIBODIES OF THE PRESENT INVENTION

Presented below is an example of the use of the monoclonal antibodies of the present invention in a diagnostic assay for HIV.

Immulon 2 plates (Dynatech) were coated with 0.5 micrograms/well of an HIV lysate diluted in 0.05M carbonate buffer, pH 9.6 for 2 hours at 37°C, and overnight at 4°C. After washing the plates with phosphate buffered saline, pH 7.4, containing 0.05% Tween (PBS-Tween), 100 microliter samples of human sera, obtained from HIV seropositive or seronegative individuals were added to each well after dilution to 1:10 to 1:1000. The plates were incubated at room temperature overnight and washed three times with PBS-Tween. A predetermined dilution of biotinylated monoclonal anti-HIV antibodies (see below) was then added in a volume of 100 microliters and the plates incubated for 2 hours at 37°C. The wells were washed three times with PBS-Tween and the reaction developed by adding an avidin-biotinylated-horseradish peroxidase complex (Vector Labs) and incubated for 30 minutes at 37°C. After washing 5 times with PBS-Tween, 2,2,-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS) was added as substrate and incubated for 30 minutes at room temperature. The optical density of each well was read in an ELISA reader at 410nm.

Biotinylation of the monoclonal antibodies to HIV was performed as follows. Each monoclonal antibody was partially purified by ammonium sulfate precipitation and/or chromatography on Protein A-Sepharose. After dialysis against 0.1M sodium carbonate, 75 microliters of N-hydroxyl-succinimidobiotin (5 mg in 1 ml of DMSO) was added to 1 ml of the antibody at a protein concentration of 5 mg/ml. The reaction was allowed to proceed at room temperature with shaking for 3 hours and then dialyzed against phosphate buffered saline, pH 7.4. The biotinylated monoclonal antibodies were stored at 25°C in 50% glycerine before use.

The results of the inhibition tests are shown in Figure 4. In Figure 4, the seronegative sera is designated (-) and the seropositive (+).

The data presented in Figure 4 show that antibodies 120-16 and 71-31 are directed against immunodominant epitopes and non-dominant epitopes respectively. Thus, seropositive sera are able to compete for the binding of labeled antibody

120-16 to the HIV lysate defining the epitope of this monoclonal antibody as immunodominant. Non-dominance was established for the epitope with which 71-31 reacts as a result of the inability of most seropositive sera to compete with this antibody for binding to the HIV lysate.

EXAMPLE 7: PRODUCTION OF THE IMMUNOTOXINS

The A chain of ricin (obtained from Island Biological, Austin, TX) was purified as described (Fulton, R.J. et al., Biol. Chem. 261: 5314-5319, 1986). Ricin (2.5 mg/ml) in 0.2M sodium acetate, pH3.5 was deglycosylated by treatment with sodium metaperiodate (10mM) and sodium cyanoborohydride (20mM) for 1 hour at 14°C as described in Blakey, D.C., et al., Cancer Res. 47: 947-982, 1987; Bourrie, B.J. et al., Eur. J. Biochem. 155: 1986. The deglycosylated A chain was then prepared from ricin holotoxin according to Fulton, A.J. et al., supra before covalent linkage.

The deglycosylated ricin A chain was covalently linked to human monoclonal antibodies 50-69 and 98-6 as described in Till, M. et al., Cancer Res. 48: 1119-1123, 1986. Briefly, N-succinimidyl-3-(2-pyrididithio) proprionate (SPDP), Sigma Chemical Co., St. Louis, MO, in dimethylformamide was added to a solution of antibody (5 mg/ml) in 0.5 M sodium phosphate buffer with 0.003 M Na_3EDTA, pH 7.5 (PBS) to give a final concentration of 1 mM. After 30 minutes at room temperature the solution was desalted on a column of Sephadex G-25 (30 X 2 mm, Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS. The derivitized protein was then mixed with the deglycosylated ricin A chain (dissolved in PBS) using 15 mg of A chain per mg of IgG and maintained for 2 hours at 25°C and overnight at 4°C. The resulting immunotoxin was purified on Sephacryl ACA-44 (Pharmacia Fine Chemicals) and evaluated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE).

The immunotoxins were analyzed under reducing and non-reducing conditions by SDS/PAGE in 10% polyacrylamide gels according to Laemmili, U.K. (Nature 223: 630-631, 1970). Protein bands were visualized by staining the gel with Coomassie Blue as is well-known in the art. Standards for the

estimation of molecular weights (obtained from Bio-Rad) were electrophoresed in the same gel. The results of such an analysis on immunotoxins constructed from human monoclonal antibodies 50-69 and 98-6 and human IgG (used as a control) are shown in Fig. 5.

In Fig. 5A & B, lane 1 is the molecular weight markers, lane 2 is human IgG (control), lane 3 is one deglycosylated ricin A chain (dgA); lane 4 is human IgG:dgA (control immunotoxin), lane 5 is immunotoxin 50-69:dgA conjugate and lane 6 is immunotoxin 98-6:dgA conjugate. In 5A the gel was electrophoresed under non-reducing conditions and in 5B the gel was electrophoresed under reducing conditions.

In Fig. 5A, two of the immunotoxins of the present invention (It-dgA) and control human IgG coupled to the deglycosylated ricin A chain (IgG:dgA) gave major bands of the predicted molecular weights (180,000 and 210,000 daltons, respectively) and a minor band of 240,000 daltons, representing 1-3 dgA chains per antibody molecule (Fig. 5A, lanes 4, 5 and 6). Free antibody was also present, but there were little or no free dgA chains. Under reducing conditions (Fig. 5B), the IgG:dgAs showed characteristic bands corresponding to heavy chain, light chain and the partially deglycosylated isomers or the A chain (A_1 and A_2, as described in Fulton, R.J. et al., supra).

The activities of the immunotoxins made from human monoclonal antibodies 50-69 and 98-6 antibodies and a control IgG:dgA were determined after reduction with dithiothreitol, a treatment which releases the dgA from the antibody molecule. The dgA's released from all three of these conjugates were comparable to unconjugated dgA in their ability to inhibit protein synthesis in a rabbit reticulocyte protein synthesis assay (IC_{50} = 1-3 x 10^{-11} M; IC_{50} is the concentration of dgA necessary to inhibit protein synthesis by 50%) (data not. shown). Each anti-HIV immunotoxin was also evaluated in a dot blot immunoassay and both retained their anti-gp41 binding activities whereas human IgG:gdA was negative (data not shown).

EXAMPLE 8: BIOLOGICAL ACTIVITY OF THE IMMUNOTOXINS PRODUCED ACCORDING TO THE PRESENT INVENTION

To determine the toxicity of the immunotoxins produced by the method of the present invention, HIV (HTLV-IIIB) infected and uninfected human cell lines H9 (T-cells, Gallo, R.C. et al., Science 224: 500-503, 1984) U937 (monocytes, Golding et al. J. Exp. Med 107:914-923, 1988) or uninfected Burkett's lymphoma cells (Daudi) were cultured with different concentrations of each experimental or control immunotoxin. Cells were used when they were in logarithmic growth. Immunotoxins (with and without chloroquine, see below) were plated in triplicate in 96-well microtiter plates in complete medium (RPMI 1640 medium, 15% (vol/vol) fetal calf serum and antibiotics). Cells were added at a final concentration of 3 x 10^5 cells per ml and plates were incubated for 36 hours at 37°C in a 5% C02/95% air atmosphere. Cells were pulse-labeled for 6 hours with 1 microCurie of [^{3}H]-thymidine and harvested with an automatic harvester [Titertek, Flow Laboratories, McLean, VI). The results are shown in Fig. 6.

In Fig. 6, results are expressed as % control (untreated cells) and are represented as follows: circles, 98-6-dgA; triangles, 50-69-dgA; squares, human Ig-dgA (control). Open symbols show experiments performed in the presence of chloroquine, closed symbols show experiments performed without chloroquine. Figures 6A and 6B depict one representative experiment of four using HIV-1-infected (6A) and uninfected (6B) H9 cells. Figures 6C and 6D show one experiment out of three (no chloroquine) and one experiment out of two (plus chloroquine) using infected (6C) and uninfected (6D) U937 cells.

In Fig. 6, it can be seen that the two immunotoxins reduced protein synthesis in H9 cells by 50% at a concentration of $2 \pm 0.8 \times 10^{-9}$M (Fig. 6A). The control immunotoxin was not toxic to infected H9 cells at concentrations of 10^{-7}M. Furthermore, neither the anti-HIV immunotoxins nor the control immunotoxin was toxic for uninfected H9 cells (Fig. 6B). The killing of HIV-infected monocytes gave an IC_{50} value of

$3 \pm 2 \times 10^{-9}$M (mean ± standard deviation in 3 experiments) but the absolute level of killing was variable (Fig. 6C, 50-80%). In order to determine if this variability was due to lysosomal degradation of the dgA prior to translocation into the cytoplasm, the above experiments were repeated using the immunotoxin-enhancer chloroquine (Ramadshman et al, Science 223: 56-61, 1984, obtained from Sigma Chemical Co.). In two experiments, chloroquine (20 micromolar) markedly potentiated the specific killing of HIV-infected U937 cells (IC_{50}=4-6 x 10^{-11}M) and resulted in a greater than 90% cell killing at 10^{-8}M. Human control immunotoxin (IgG:dgA) was not toxic for HIV-infected cells in the presence or absence of chloroquine and neither of the specific anti-HIV immunotoxins was toxic to uninfected U937 cells with or without chloroquine.

To further demonstrate the specificity of cell killing, a concentration of immunotoxin 98-6:dgA or immunotoxin 50-69:dgA that would kill 74-82% of infected H9 cells (5 micrograms per ml) was used in the presence of recombinant gp160, (which is cleaved in HIV-infected cells to yield gp120 and gp41) recombinant gp120, purified, unconjugated monoclonal antibodies 50-69, 98-6 or control human IgG at concentrations of 0.25 to 25 micrograms per ml. The results are shown in Table IV and were calculated as follows:

$$\% \text{ killing} = \left[100 \times \frac{\text{cpm cells + It-dgA + blocker}}{\text{cpm cells in media only}}\right]$$

TABLE IV

Blocker	Blocker conc. (micrograms/ml)	98-6:dgA Exp. 1	98-6:dgA Exp. 2	50-69:dgA Exp. 1	50-69:dgA Exp. 2
None	--	72	73	73	74
rgp160	25	33	0	7	6
	2.5	62	8	76	77
	0.25	79	75	76	75
rgp120	25	80	73	76	74
	2.5	82	77	76	73
	0.25	81	78	76	75
98-6	25	49	29	37	30
	2.5	76	67	75	67
	0.25	82	75	76	73
50-69	25	36	13	25	57
	2.5	70	31	70	74
	0.25	80	71	76	79
Human IgG (control)	25	79	82	77	80
	2.5	79	77	77	77
	0.25	75	71	71	74

As shown in Table IV, unconjugated monoclonal antibodies 50-69 and 98-6 partially blocked the cell killing by their respective immunotoxins whereas recombinant gp160 was a highly effective blocker. Normal human IgG and recombinant gp120 did not block the killing. These data show that the anti-gp41:dgA are cytotoxic because of their specificity for gp41.

Since gp41 may have homology with class II histocompatibility antigens, the effects of the two anti-gp41 immunotoxins on class II+ Daudi cells was examined. The immunotoxins were not toxic to Daudi cells at concentrations as high as 10^{-7}M ($IC_{50}=10^{-7}$M) although an anti-class II immunotoxin (manufactured with an anti-class II monoclonal antibody as described above) had an IC_{50} of $1.2 \pm 0.2 \times 10^{-10}$M (mean $\pm$ standard deviation) in three experiments, showing that the cells were not merely refractory to cell killing by the dgA (data not shown).

WHAT IS CLAIMED IS:

1. An immunotoxin comprising:
a conjugate of a human monoclonal antibody directed against human immunodeficiency virus gp41, said antibody being of the IgG isotype, and
the A chain of ricin.

2. The immunotoxin of claim 1 wherein said A chain is deglycosylated.

3. An immunotoxin comprising:
a conjugate of a human monoclonal antibody directed against human immunodeficiency virus p24, said antibody being of the IgG isotype, and
the A chain of ricin.

4. The immunotoxin of claim 3 wherein said A chain is deglycosylated.

5. A pharmaceutical formulation for treating a mammal suffering from an infection caused by HIV comprising an effective amount of an immunotoxin comprising a human monoclonal antibody directed against human immunodeficiency virus gp41 and the A chain of ricin, wherein said antibody is of the IgG isotype.

6. The pharmaceutical formulation of claim 5 wherein said A chain is deglycosylated.

7. The pharmaceutical formulation of claim 6 further comprising an effective amount of an immunotoxin potentiator.

8. A pharmaceutical formulation for treating a mammal suffering from an infection with HIV comprising a conjugate of a human monoclonal antibody directed against human immunodeficiency virus p24 and the A chain of ricin, wherein said antibody is of the IgG isotype.

9. The pharmaceutical formulation of claim 8 wherein said A chain is deglycosylated.

10. A method for treating a mammal suffering from an infection caused by HIV comprising administering to a mammal in need of such treatment an effective amount of an immunotoxin comprising a conjugate of human monoclonal antibody directed against human immunodeficiency virus gp41 and the A chain of ricin, wherein said antibody is of the IgG istype.

11. The method of claim 10 wherein said A chain is deglycosylated.

12. The method of claim 10 also comprising administering an effective amount of an immunotoxin potentiator.

13. The method of claim 12 wherein said immunotoxin potentiator is chloroquine.

14. A method for treating a mammal suffering from an infection caused by HIV comprising administering to a mammal in need of such treatment an effective amount of an immunotoxin comprising a conjugate of a human monoclonal antibody directed against human immunodeficiency virus p24 and the A chain of ricin, wherein said antibody is of the IgG istype.

15. The method of claim 14 wherein said A chain is deglycosylated.

16. The method of claim 15 comprising also administering an effective amount of an immunotoxin potentiator.

17. The method of claim 16 wherein said immunotoxin potentiator is chloroquine.

18. An immunotoxin comprising:
a conjugate of the human monoclonal antibody produced by cell line CRL 10037, and
the deglycosylated A chain of ricin.

19. An immunotoxin comprising:
a conjugate of the human monoclonal antibody produced by cell line CRL 10038, and
the deglycosylated A chain of ricin.

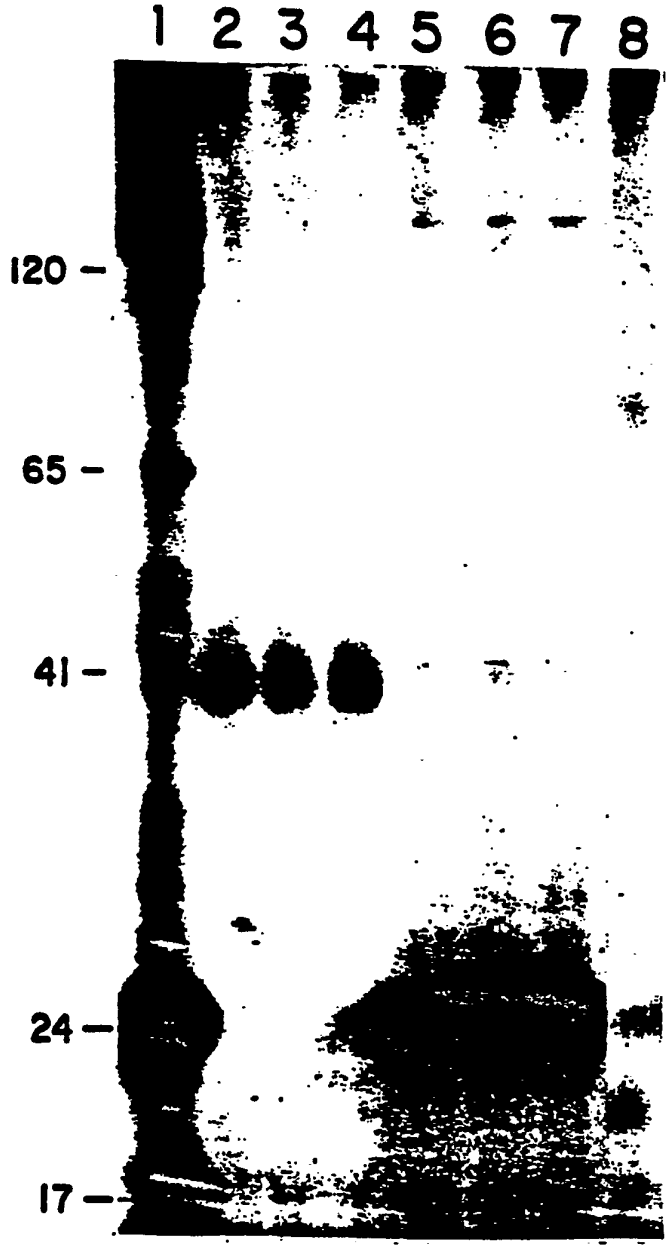


Fig 1

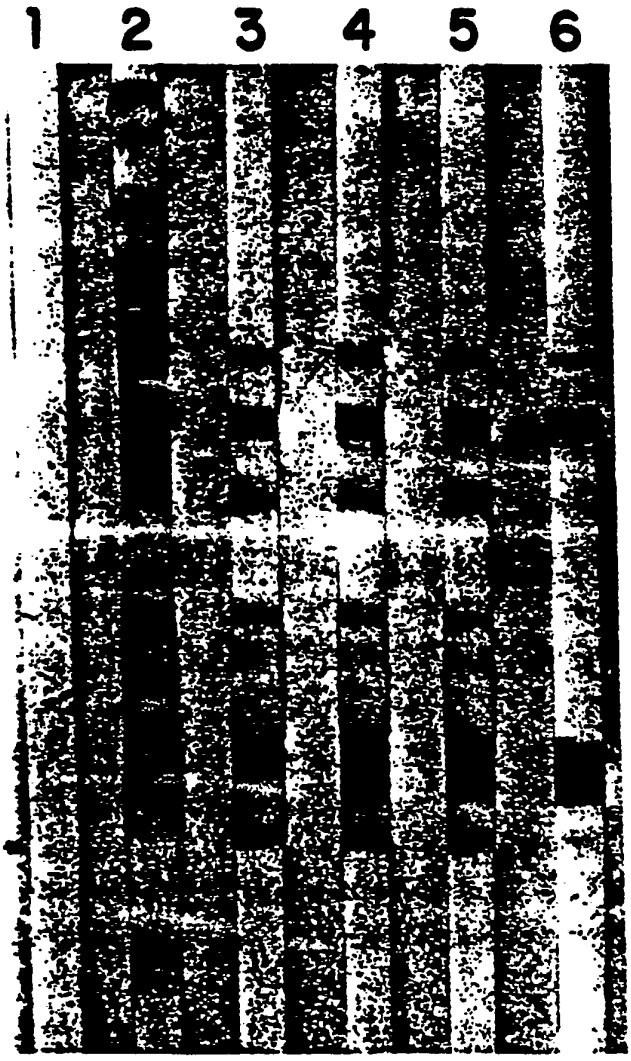
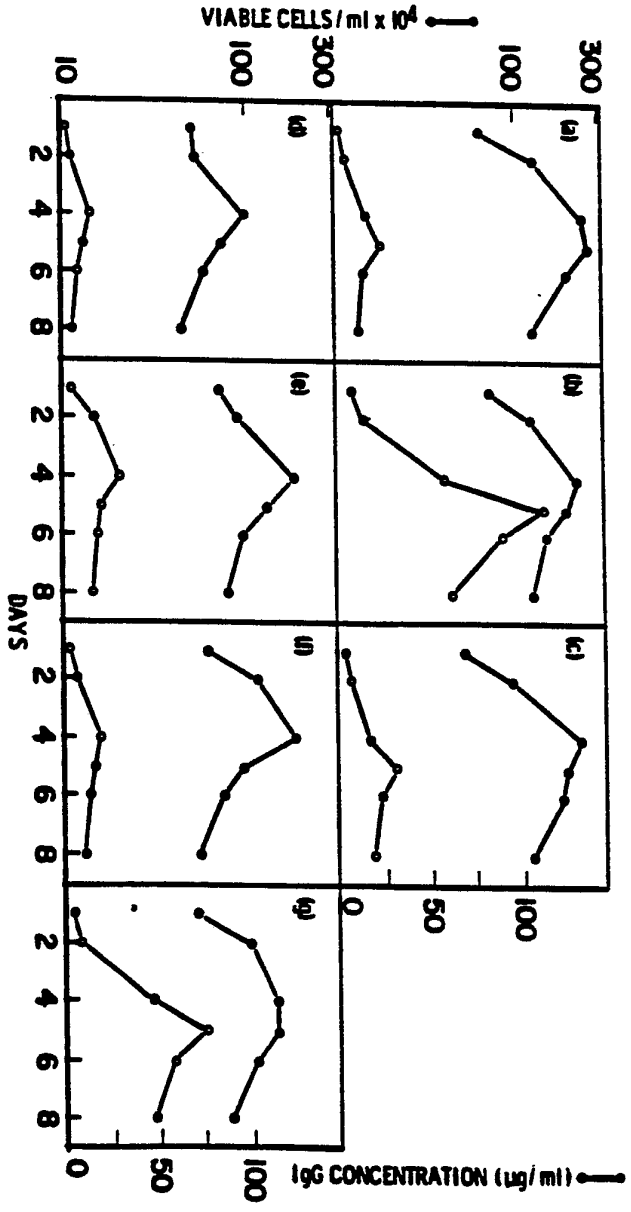


Fig 2

Fig 3


VIABLE CELLS / ml x 10⁴
10
100
300
100
300
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
DAYS
2
4
6
8
0
50
100
IgG CONCENTRATION (µg/ml)

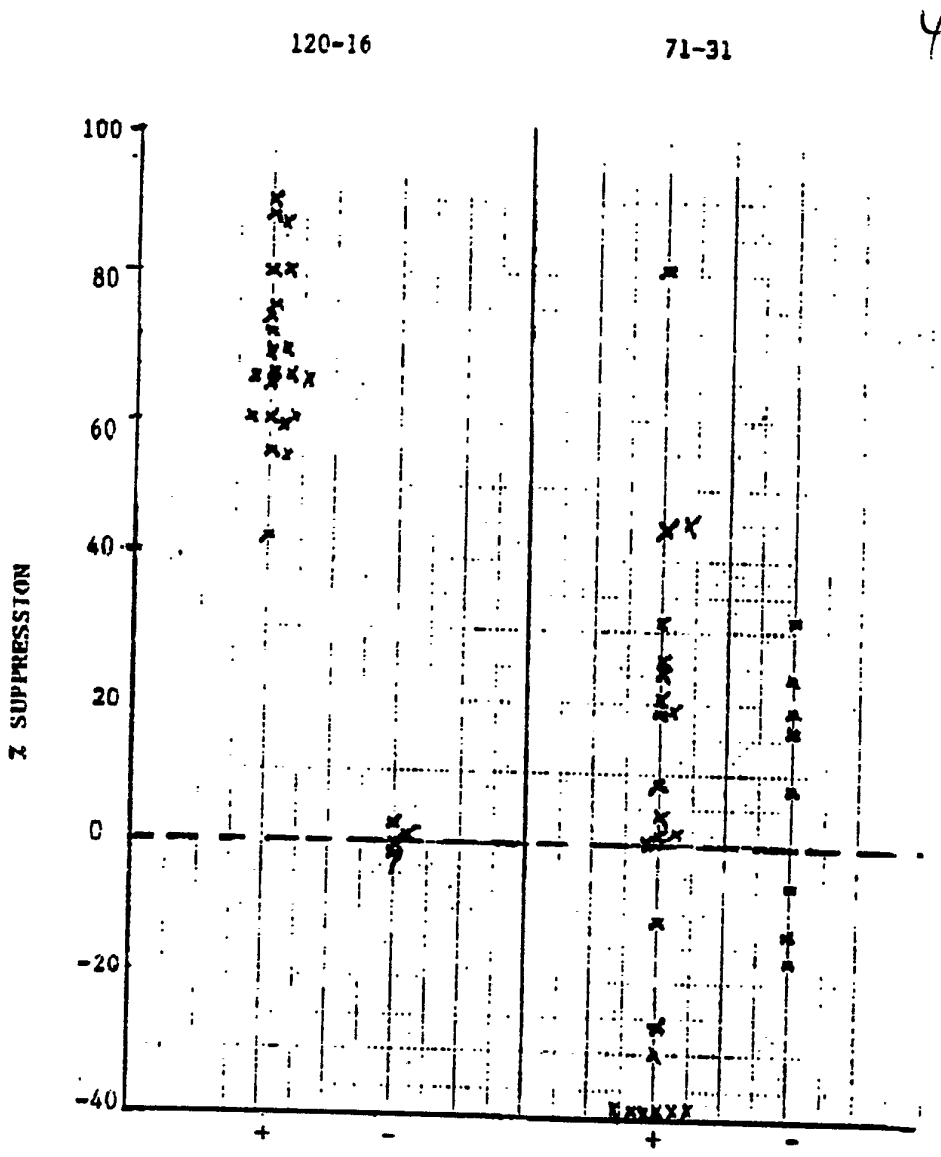


Fig 4

5/6

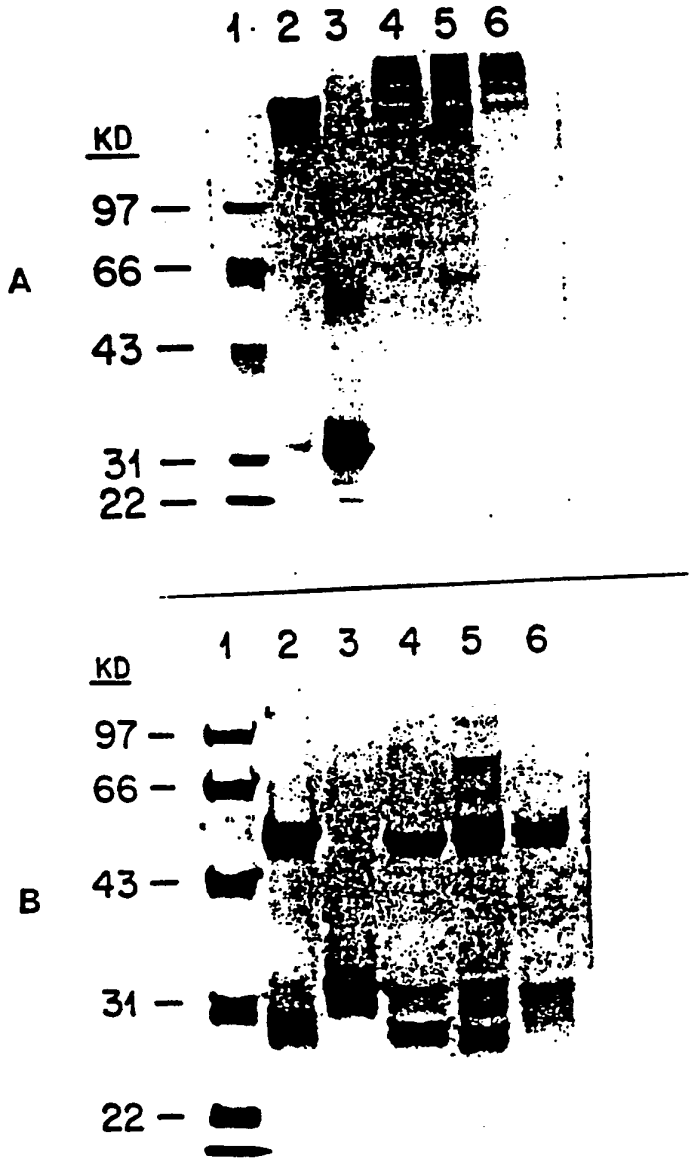


Figure 5

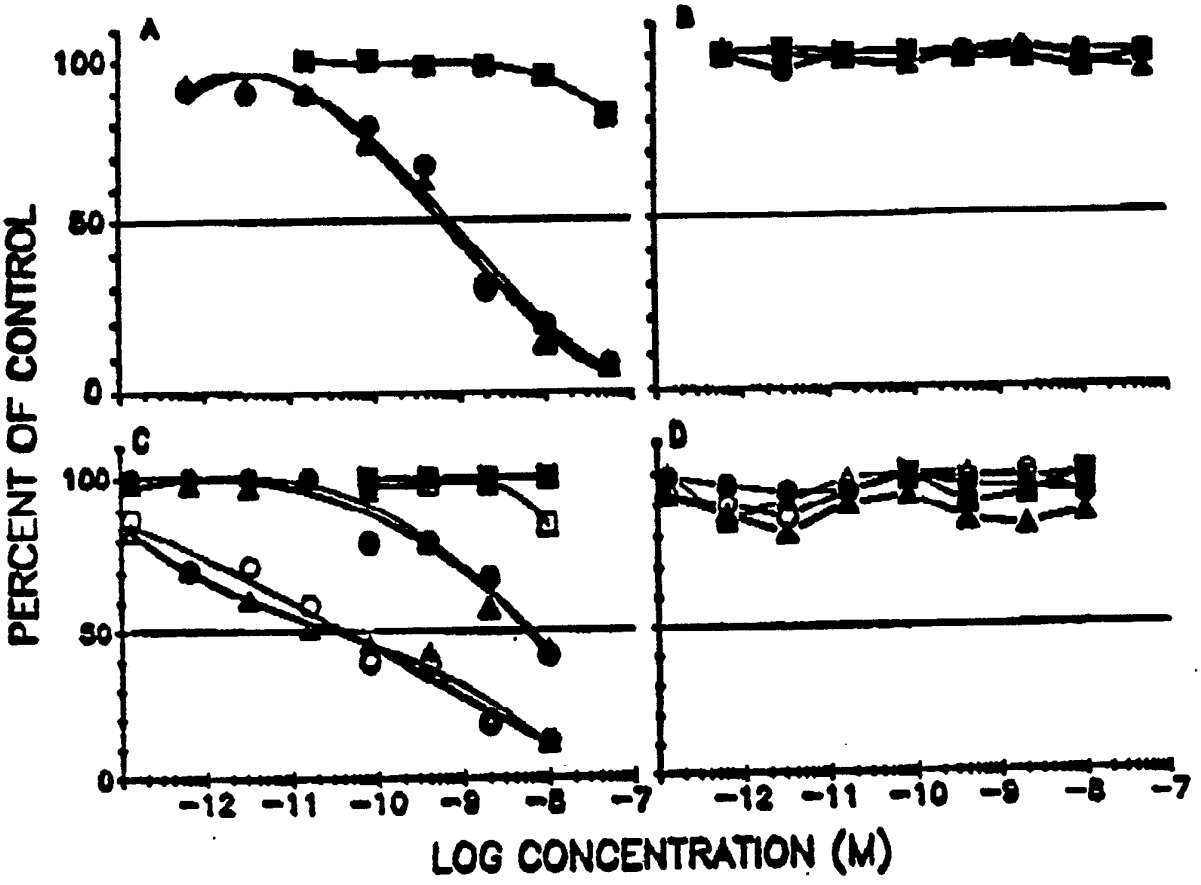

A
B
C
D
100
50
0
PERCENT OF CONTROL
-12 -11 -10 -9 -8 -7
LOG CONCENTRATION (M)

Figure 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01396

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

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

IPC(5): A61K 39/00; 39/44; 37/00

USCl.: 424/85.8, 85.91; 530/387, 389, 390, 391

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/85.8, 85.91; 530/387, 389, 390, 391

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Databases: Chemical Abstracts Services Online (File CA, 1967-1990); File Giosis, 1969-1990). Automated Patent System (File USPAT, 1975-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	EP, A, 0251612, 07 JANUARY 1988, McClure, See pages 3-5.	1,5,10,18 2-4,6-9,11-17, 19
Y	Journal of Immunological Methods, Vol. 106, 1988, Grunow et al., "The high efficiency, human B cell immortalizing hetromyeloma CB-F7", see pages 257, 258 and 263.	1-19
Y,P	Proceedings National Academy Sciences, Vol. 86, March 1989, Gorny et al., "Generation of human monoclonal antibodies to human immuno-deficiency virus", see page 1624.	1-19
Y	The Lancet, 23 APRIL 1988, Desgranges et al., "Monoclonal antibodies to HIV in a non-infected, immunized donor", see pages 935-936.	1,5,10,18 2-4,6-9,11-17, 19
X,P Y,P	Proceedings National Academy Sciences, Vol. 86., March 1989, Till et al., "Human immunodeficiency virus-infected T cells and monocyles are killed by monoclonal human anti-gp41 antibodies coupled to ricin A chain".	1,2,5-7,10-12, 18 3,4,8,9,13-19

* Special categories of cited documents: [15]

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"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.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
08 JUNE 1990	26 JUL 1990
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	for Andrew Robinson D. Bernstein

Form PCT/ISA/210 (second sheet) (May 1986)

cdb:6/30/90

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X,P Y,P	The Journal of Immunology, Vol. 142, 01 MAY 1989, Pincus et al., "Treatment of HIV tissue culture infection with monoclonal antibody- ricin A chain conjugates", see page 3070.	1,5,10,18 2-4,6-9,11-17, 19
X,P Y,P	Journal of Virology, Vol. 63, No. 6, June 1989, Pinter et al, "Olgomeric structure of gp41, the transmombrane protein of human immunodeficiency virus type 1", see page 2674.	1,5,10,18 2-4,6-9,11-17, 19
Y	US, A, 4,582,703, (JANSEN et al.) 15 APRIL 1986.	1-19