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(54) Title: PROPHYLACTIC AND THERAPEUTIC CONTROL OF RETROVIRAL INFECTIONS

(57) Abstract

The present invention provides a method of inducing immunity to retroviral infection in a human or other mammal comprising administration of a preparation comprising retroviral antigen in an amount effective to induce a cell mediated immune response substantially free of a humoral immune response. There is also provided a vaccine effective against HIV. In one aspect, the immunogen is useful for immunizing an individual previously infected by a retrovirus including HIV, so as to induce immunoprotective factors protective against progression of the infection. In another aspect, the vaccine is useful for vaccinating an individual not previously infected with HIV in order to prevent subsequently acquired infection. In another aspect, there is provided a method of rendering a viral immunogen non-infectious.

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PROPHYLACTIC AND THERAPEUTIC CONTROL OF RETROVIRAL INFECTIONS

BACKGROUND OF THE INVENTION

This invention relates generally to compositions and methods for the prevention and treatment of retroviral disease and more specifically to compositions for the prevention and treatment of Human Immunodeficiency Virus (HIV) infection. The method comprises the use of HIV antigen in vaccinations against and immunotherapy of Human Immunodeficiency Virus infected patients.

Acquired Immune Deficiency Syndrome, also known as AIDS, has been described as a modern plague. In the years since its first description in 1981, it has claimed millions of victims, and accounted for tens, if not hundreds of thousands of deaths in the United States alone. However, the true impact of the disease has yet to be felt. The virus may remain latent in infected individuals for five or more years before symptoms appear. Many Americans may unknowingly be infected and capable of infecting others who might come into contact with their body fluids. Thus, if unchecked, the personal, social and economic impact of AIDS will be enormous.

The causative agent of AIDS is the retrovirus Human Immunodeficiency Virus (HIV). Retroviruses are distinguished by the fact that their genetic material, which is RNA, is reverse transcribed during the viral replication process. Upon infection of a host cell, the RNA acts as a template for the reverse transcription to DNA, catalyzed by an enzyme called reverse transcriptase. The DNA so produced enters the cell nucleus where it is integrated into the host DNA as a provirus. When properly activated, the retroviral-derived DNA is transcribed and translated to produce RNA containing virions which are then released from the cell by a budding process.

Certain viruses, including retroviruses, may remain in a latent state for months or years before they are activated and virions are produced. Although asymptomatic, a host may nonetheless harbor the virus in a proviral form, thus being potentially at risk of disease and of infecting others.

When an individual becomes infected with HIV, the virus preferentially attaches to and enters a particular class of cells termed T4 lymphocytes, characterized by the presence of a cell surface marker termed CD4. These white blood cells play an integral role in the immune system, functioning as critical components of both the humoral and cellular immune response. Much of the deleterious effect of HIV can be attributed to the functional depression or destruction of T4 lymphocytes.

The intact HIV virion is roughly spherical and is approximately 110 nm in diameter. The virion has an outer membrane covered with knobs or spikes made up of glycoprotein, gp160/120. In addition, there exists a transmembrane protein termed gp41. Inside the virion are the structural proteins: an outer shell is composed of the protein p17 and an inner nucleoid or central core is made up of the phosphoprotein, p24. The viral RNA is present inside the core along with two copies of the reverse transcriptase enzyme, p66/51, which is necessary for the synthesis of viral DNA from the RNA template.

The HIV RNA genome encodes three major structural genes: gag, Pol and env, which are flanked at either end by long terminal repeat (LTR) sequences. The gag gene codes for the group-specific core proteins, p55, p39, p24, p17 and p15. The Pol genes code for the reverse transcriptase p66/p51 and the protease p31. The env genes encode the outer envelope glycoprotein gp120 and its precursor gp160 and the transmembrane glycoprotein gp41. Some of the genes tend to be highly variable, particularly the env genes. In addition, there are five other genes, not shared by other retroviruses, which are either involved in transcriptional or translational regulation or encode other structural proteins. The entire HIV genome has now been sequenced. See Ratner et al. Nature 313:277 (1985) which is incorporated herein by reference.

The HIV attaches to host cells by an interaction of the envelope glycoproteins with a cell surface receptor. It appears that when HIV makes contact with a T4 cell, gp120 interacts with the CD4 receptor. The viral

envelope is then fused with the cell membrane and the inner core of the virus enters the infected cell where the transcription of RNA into a DNA provirus is catalyzed by reverse transcriptase. The provirus may remain in the cell in a latent form for some months or years, during which time the infected individual is asymptomatic. However, if the virus is later activated causing viral replication and immunosuppression the individual will then be susceptible to the opportunistic infections, including cancer, associated with AIDS.

As yet, no vaccine or treatment is known which has been proven completely effective against the AIDS syndrome. Attempts to develop vaccines have centered on attempts to induce high antibody titers to the HIV antigens. Certain antibodies reactive with HIV, notably anti-gp160/120 and virus neutralizing antibodies, are present at high levels throughout both the asymptomatic and symptomatic phases of the HIV infection. It has been suggested that, rather than playing a protective role, such antibodies may in fact promote the attachment and penetration of the virus into the host cell.

There are two broad classes of immune responses: (1) **Cell-mediated immune responses** involve the production of specialized cells that react mainly with foreign antigens on the surface of host cells, either killing the host cell if the antigen is an infecting virus or inducing other host cells, such as macrophages, to destroy the antigen. (2) **Humoral antibody responses** involve the production of antibodies, which circulate in the bloodstream and bind specifically to the foreign antigen that induced them. The binding of antibody to the antigen makes it easier for phagocytic cells to ingest the antigen and often activates a system of blood proteins, collectively called *complement*, that helps destroy the antigen.

Some HIV antigens induce primarily cell mediated immunity whereas others can, under different conditions, induce either cell-mediated or humoral immunity. There is a tendency for the immune response to an antigen, at a particular time, to be exclusively of the cell-mediated or humoral type.

There are two components involved in T-helper cell function, referred to as TH-1 and TH-2, which result respectively in a dominance of a CMI/DTH or an antibody response. TH-1 cells are primarily responsible for cell-mediated immunity although TH-1 cells are believed to stimulate the production of a

certain antibody isotype, IgG2a. TH-2 cells are primarily responsible for humoral immunity. TH-1 cells, upon activation produce certain cytokines, such as IFN-γ which interferes with one or more aspect of TH-2 cell development and function. TH-2 cells produce certain cytokines such as IL-4 and IL-10 which conversely can inhibit TH-1 cell development and function. The objective of most immunization protocols has been to provide the greatest amount of humoral immunity, i.e., the highest antibody titer achievable. However, recent observations suggest that a high antibody titer may not confer immunity to HIV infection, and in fact, may speed the progression of the conversion of HIV seronegativity to HIV seropositivity.

Many non replicating antigens can induce either delayed-type hypersensitivity (DTH)/Cell-mediated immunity (CMI) or antibody-mediated hypersensitivity, depending on quantitative variables such as antigen doses and/or qualitative variables such as type of antigen, addition of and type of adjuvant administered. A concentration of antigen subimmunogenic for the induction of antibody can often induce DTH (CMI). Bretcher et al. (in press) have demonstrated that an antibody unresponsive state may be induced in leishmania susceptible mice by injection of subimmunogenic doses of live leishmania organisms. This subimmunogenic dose confers resistance to subsequent higher dose challenges with the Leishmania organism. This state of unresponsiveness for the induction of antibody is associated with the expression of DTH to the live antigenic organism. This type of immune response is probably associated with the induction of antigen-specific CD8+ T cells that suppress the induction of the humoral response. Thus, it may be possible to manipulate the immune system by manipulating the immunization regimen so that the immune response to the particular antigen is locked into a cell-mediated mode. One method for achieving this comprises administering concentrations of antigen subimmunogenic for the induction of antibody

There thus exists a need for an immunization method useful in the prevention and therapy of retroviral infections, particularly those attributed to HIV. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a method of inducing immunity to retrovirus infection in a human or other mammal comprising administration of a preparation comprising a retroviral antigen in an amount effective to induce a prolonged cell-mediated immune response while preventing or delaying the development of a humoral response. Preferably, the method provides the antigen preparation in an amount which induces a CMI response substantially free of a humoral immune response. The retroviral antigen may be inactivated retrovirus particles or antigenic components thereof. Preferably, the antigen comprises a non-infectious immunogen containing retroviral particles which may be free of outer envelope proteins or containing selected antigens isolated from a retrovirus. However, when the antigen is genetically engineered into a vector the vector itself may be infectious, but not pathogenic. In one aspect, the immunogen is useful for immunizing an individual infected by a retrovirus, so as to induce immunoprotective factors protective against progression of the infection. In another aspect, the immunogen is useful for vaccinating an individual not previously infected in order to prevent subsequent infection. The antigen preparation may be administered alone, or in conjunction with an adjuvant which favors the production of CMI over a humoral immune response.

The retroviral antigen , preferably HIV, can be administered at any dosage sufficient to induce a cell mediated response but subimmunogenic for the induction of antibody. Preferably the dosage of HIV antigen is no larger than from about 100 µg to about 200 µg, and may be orders of magnitude smaller. Alternatively, a higher dosage of HIV antigen may be used in conjunction with one or more other therapeutics which prevent or diminish antibody induction but do not prevent or diminish the development of the induction of cell-mediated immune response to HIV antigen. For instance, cyclosporin A prevents or diminishes the humoral immune response and may be administered concurrently with the retroviral, preferably HIV, antigen preparation in a special embodiment of the method of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates the mean scores of a skin test utilizing 10 µg HIV and 3 adjuvant formulations.

Figure 2 demonstrates the individual animal data of a skin test utilizing 10 µg HIV and 3 adjuvant formulations.

Figure 3 demonstrates the results of an antigen proliferation assay for cell mediated immunity.

Figure 4 demonstrates the mean scores of the antibody titers from an r-p24 ELISA.

Figure 5 demonstrates the antibody titers from an r-p24 ELISA.

Figure 6 demonstrates the results of antibody determination by Western Blot.

Figure 7 demonstrates the mean scores of the antibody determined by Western Blot.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an effective means for the prevention of retroviral infection and for the post-exposure treatment for arresting progression of retroviral infections. A special embodiment of this invention comprises the effective means for the prevention of HIV caused AIDS Related Complex (ARC) and AIDS and for the post-exposure treatment for arresting progression of HIV infections. Individuals who have been exposed to the HIV virus express in their serum certain antibodies specific for HIV. Such individuals are termed "seropositive" for HIV, in contrast to individuals who are "seronegative." The presence of HIV specific antibodies can be determined by commercially available assay systems. The level of these antibodies is indicative of the progression of the AIDS syndrome.

High levels of anti-gp160/120 (outer envelope) antibody, which are present in the asymptomatic phase of the HIV infection also persist in the symptomatic phase. The level of anti-p24 antibody in the asymptomatic phase is high but appears to decline in the symptomatic phase. Similarly, HIV seropositive sera contain an antibody that inhibits the function of reverse transcriptase (anti-RT antibody or RTI). In individuals in whom RTI is present at

high levels, attempts at virus isolation are less frequently positive than in those in whom it is absent. It therefore appears that the decline of cellular and humoral immunoprotective factors such as T4 cells and anti-GAG antibodies, including anti-p24, and anti-POL antibodies, including RTI, is associated with progression of the HIV infection to AIDS.

The production of immunoprotective factors may be induced by vaccinating an individual with an effective immunogen. In the past, attempts have been made to develop a vaccine based on certain viral proteins, for example the envelope proteins. The present invention relates to an immunogen containing HIV gene products which may exclude the outer envelope proteins which is useful in stimulating the production of immunoprotective factors effective for preventing infection in non-infected individuals and for slowing or preventing the progression of the HIV infection to AIDS in infected individuals. The immunogen comprises intact viral particles from which the outer envelope has been removed, or comprises one or more HIV gene products other than gp120 or gp160.

A. Definitions

As used herein, the term "retrovirus" refers to a virus having as its genetic material ribonucleic acid (RNA) which is transcribed into DNA which is inserted into the host genome. Examples of retroviruses include HTLV-I, HTLV-II STLV-I, and the lentivirus family including HIV, visna virus, equine infectious anemia virus, feline immunodeficiency virus and bovine immunodeficiency virus. These are described in Fauci, Science 239:617 (1988), and references included therein, each of which are incorporated herein by reference.

As used herein, the term "HIV" includes types 1 and 2 and is synonymous with HTLV-III and LAV-I and LAV-2. HIV refers to the virus generically and includes all forms, subtypes and variations. Various cell lines permanently infected with the HIV virus have been developed and deposited with the ATCC including those having accession numbers CCL 214, TIB 161, CRL 1552 and CRL 8543, all of which are described in U. S. Pat No. 4,725,669 and Gallo, Scientific American 256:46 (1987) each of which are incorporated herein by reference.

As used herein, the term "outer envelope protein" refers to that portion of the membrane glycoprotein of a retrovirus which protrudes beyond the membrane, as opposed to the transmembrane protein, gp41. The outer envelope protein is, in HIV, synonymous with gp120 and its precursor gp160.

As used herein, the term "outer envelope free" refers to a preparation of retroviral particles or retroviral gene products devoid of the outer envelope proteins.

As used herein, the term "gp120" or "gp160/120" refers to glycoproteins having either the antigenic specificity or the biological function of the outer envelope protein; gp160 is believed to be the precursor of gp120.

As used herein, "AIDS" refers to Acquired Immune Deficiency Syndrome as described by Adler, Brit. Med. J. 294: 1145 (1987). It is characterized by tumors and a series of opportunistic infections. "ARC" refers to AIDS-Related Complex as described by Adler, Supra. The symptomatic phase of HIV infection refers to the onset of symptoms characteristic of ARC or AIDS. The "AIDS syndrome" includes both AIDS and ARC.

As used herein, the term "gene product" refers to a polypeptide or protein which is encoded by a gene. The term is intended to include protein derivatives such as glycoproteins. It is understood that limited modifications may be made in the amino acid sequence of the gene product without destroying the biological function or immunogenicity of the gene product and that only a portion of the primary sequence may be required for immunogenicity.

As used herein, the term "HIV infected" or "HIV noninfected" refer to humans or other mammals in whom the HIV virus or provirus is present, or is not present, respectively. "Retrovirus infected" or "retrovirus non-infected" refer to individuals in whom a retrovirus or provirus is present, or is not present, respectively. At the present time, serological tests to detect the presence of antibodies to the virus are the most widely used method of determining infection. Such methods can, however, result in both false negatives, as where an individual has contracted the virus but not yet mounted an immune response, and in false positives, as where a fetus may acquire the antibodies, but not the virus from the mother. Other methods of determining the presence of the virus

have or may become available. Where serological tests provide an indication of infection, it may be necessary to consider all those who test seropositive as in fact, being infected. Further, certain of those individuals who are found to be seronegative may in fact be treated as being infected if certain other indications of infection, such as contact with a known carrier or the presence of anti-HIV CMI, are satisfied. While the terms "seropositive" and "seronegative" may be used herein as the most readily available indicators of infection, they are intended to be synonymous with "infected" or "non-infected" respectively. Identification of HIV specific genes and gene products is based on the terminology of HIV type 1. It is intended, however, that a reference to a specific gene or gene product of HIV type 1, based on its molecular weight, will also include the corresponding gene or gene product of HIV type 2, and, where an homologous gene is present, of other retroviruses. The gene products of other types and species may have slightly different molecular weights. For example, HIV type 1 gp41 is equivalent to gp36 of type 2, while type 1 gp120 corresponds to type 2 gp 130. Genes are identified by iunderlined lower case designations, such as gag, while the corresponding gene product is identified by an upper case designation, such as GAG. The entire HIV gene has been sequenced. See Ratner et al. Nature 313:277(1985), which is incorporated herein by reference.

In order to isolate virus, mononuclear cells from peripheral blood such as lymphocytes, can be obtained by layering a specimen of heparinized venous blood over a Ficoll-Hypaque density gradient and centrifuging the specimen. The mononuclear cells are then collected, activated, as with phytohemagglutinin for two to three days, and cultured in an appropriate medium, preferably supplemented with interleukin 2. The virus can be detected either by an assay for reverse transcriptase, by an antigen capture assay for p24, by immunofluorescence or by electron microscopy to detect the presence of viral particles in cells all of which are methods well-known to those skilled in the art. Once isolated, the virus can be transmitted to other cells.

It is important to use a non-infectious viral vaccine in order to avoid introducing the viral infection into a host. Various methods are well known for rendering a pathogen non-infectious. See for example Hanson, MEDICAL VIROLOGY II (1983) (de la Maza and Peterson, eds) Elsevier, N.Y. The virus may be inactivated or made replication-defective. Preferably, however, it is

treated with a combination of beta-propiolactone and gamma radiation. For the amounts used herein, the beta-propiolactone must be in contact with the virus for a minimum of 2.5 hours. In order to completely eliminate any residual beta-propiolactone, the beta-propiolactone must remain in solution for a minimum of five hours at 37°C.

Preferably, the isolated virus is then treated so as to remove the outer envelope proteins. Such removal is preferably accomplished by repeated freezing and thawing of the virus in conjunction with physical methods which cause the swelling and contraction of the viral particles, although other physical or non-physical methods, such as sonication, can also be employed alone or in combination.

Alternatively, substantially purified gene products of retrovirus such as HIV, other than the outer envelope proteins can be used as an immunogen. Such gene products include those products encoded by the gag genes (p55, p39, p24, p17 and p15), the pol genes (p66/p51 and p31-34) and the transmembrane glycoprotein gp41. These gene products may be used alone or in combination. Alternatively, the gene products of the remaining five genes of the HIV genome may be used. The gene products may be isolated and purified from the virus or may be produced by cloning and expressing the appropriate gene in a host organism such as bacterial, fungal or mammalian cells, by methods well known in the art. Alternatively, the antigens may be synthesized, using methods well known in the art, such as automated peptide synthesis. The amino acid sequence of the gene products has been deduced from the nucleotide sequence.

The term "antigen preparation" is meant to comprise any composition, including living organisms used as vectors, comprising any single retroviral antigen, preferably HIV antigen, any combination of retroviral, preferably HIV, antigens, and includes, additionally, inactivated, mutated or otherwise manipulated retroviral, preferably HIV, virus or viral antigens, utilized alone or in combination with an adjuvant to assist or direct the type of immune response which results from administration of the immunogen. The term "antigen preparation" is meant to also include genetically engineered molecules comprised of one or more retroviral, preferably HIV, antigen sites. Additionally,

said genetically engineered molecules may additionally contain one or more adjuvants.

The antigen preparation may comprise the retroviral antigen in a suitable pharmaceutical carrier. The antigen preparations of this invention can be normally administered intradermally, subcutaneously, intramuscularly, in the treatment of patients having retroviral infections or to prevent retroviral infections. As used herein, the term "patient" includes humans and other mammals.

The antigen preparations of this invention may be formulated for administration in any convenient way, and the invention includes within its scope pharmaceutical compositions containing at least one antigen preparation according to the invention adapted for use in human or veterinary medicine. Such compositions may be formulated in a conventional manner using one or more pharmaceutically acceptable carriers or excipients. Suitable carriers include diluents or fillers, sterile aqueous media and various non-toxic organic solvents. The compositions may be formulated in the form of injectable solutions and the like and may contain one or more agents in order to provide a pharmaceutically acceptable preparation. Additionally, said antigen preparation may comprise other compositions including adjuvants and other therapeutic pharmaceutical compounds such as cyclosporin .

The particular carrier and the ratio of the antigen preparations to carrier are determined by the solubility and chemical properties of the compounds, the particular mode of administration and standard pharmaceutical practice. For example, excipients such as lactose, sodium citrate, calcium carbonate and dicalcium phosphate and various disintegrants such as starch, alginic acid and certain complex silicates, together with lubricating agents such as magnesium stearate, sodium lauryl sulphate and talc, can be used in producing tablets. For a capsule form, lactose and high molecular weight polyethylene glycols are among the preferred pharmaceutically acceptable carriers. Where aqueous suspensions for oral use are formulated, the carrier can be emulsifying or suspending agents. Diluents such as ethanol, propylene glycol, glycerin and chloroform and their combinations can be employed as well as other materials.

For parenteral administration, solutions or suspensions of these compounds in sesame or peanut oil or aqueous propylene glycol solutions, as well as sterile aqueous solutions of the soluble pharmaceutically acceptable salts described herein can be employed. Solutions of the salts of these compounds are especially suited for administration by intramuscular and subcutaneous injection. The aqueous solutions, including those of the salts dissolved in pure distilled water, are suitable for administration by intravenous injection, provided that their pH is properly adjusted, and that they are suitably buffered, made isotonic with sufficient saline or glucose and sterilized by heating or by microfiltration.

The dosage regimen used in carrying out the methods of this invention is that which insures maximum CMI response while provoking the minimal humoral response. Thus, in general, the dosages are those that are therapeutically effective in producing a CMI response, but subtherapeutic in producing a humoral immune response. In general, the injectable dose may be between about 0.1 and about 200 μg (preferably in the range of about 100 to less than 200 μg), bearing in mind, of course, that in selecting the appropriate dosage in any specific case, consideration must be given to the patient's weight, general health, age and other factors which may influence response to the antigen preparation. The amount of antigen in the antigen preparation may vary depending on the presence and type of adjuvants or humoral immune response inhibiting agents. For instance, the dose of HIV antigen which, in the presence of an adjuvant such as IFA, would result in a humoral response, may induce ,no humoral response in the presence of a different adjuvant , such as Ribi Corps DETOX or NAGO as described by Zheng, et al., (1992. *Science.* 256:1560).

The compounds of the invention may be administered as frequently as is necessary to achieve and sustain the desired therapeutic response. Some patients may respond quickly to a relatively large or small dose and require little or no maintenance dosage. On the other hand, other patients may require sustained dosing to prolong a CMI response.

A subset of individuals determined to have retroviral infections, such as HIV, can be effectively treated by active immunotherapy using a non-infectious immunogen prepared from the retrovirus. Animals known to have a retroviral infection can also be treated by such a method. In the case of HIV, a

seropositive individual is immunized with an outer envelope free immunogen, preferably incorporated in an adjuvant. Alternatively, the immunogen can be administered in its aqueous form without an adjuvant. The dose is selected so as to be immunologically effective to induce a maximal cell mediated immune response, while inducing a minimal humoral immune response , and is generally between about 100 to about 200 μg of protein, preferably about 100 μg of protein.

Preferably, the immunoresponsiveness or immunocompetence of the individual is determined prior to immunization in order to determine an appropriate course of therapy. As a method of such determination, individuals' sera are screened for the presence of antibodies to p24 (as by means of ELISA), for RTI antibody and/or for the level of T4 cells and cell mediated immunocompetence by methods well known in the art. Methods for assaying the presence of antibodies may be any known to those of skill in the art, for instance the ELISA described by Gibbs, et al. (1991. *Proc. Natl. Acad. Sci. USA* 88:3348). Assays to measure cell mediated immunity such as delayed type hypersensitivity are also known to those in the art, such as those described in Peterson, et al.; 1983. *J. Allergy Clin Immunol.* 71:612; Gordon, et al.; 1983. *J. Allergy Clin. Immunol.* 72:487; and Corriel, et al.; 1985. *Am. J. Dis. Child.* 139:141.

Seronegative individuals can be vaccinated in order to induce immunoprotective factors to prevent infection. Preferably, the vaccine is administered initially by intramuscular injection followed by a booster injection given either intramuscularly or intradermally. A physiologically effective dose, preferably in the range of about 1 to about 200 μg and more preferably about 100 μg of immunogen is provided per dose. Preferably the vaccine is administered in conjunction with an adjuvant, most preferably a water-in-oil type adjuvant. Adjuvants are generally used to enhance the ability of immunogens to elicit an immune response. Aluminum salts (alum) were first described as adjuvants over 60 years ago, and they remain the only agents approved for human use. Aluminum salts have limited potency and do not consistently enhance cell-mediated responses, so improved adjuvants are being developed. Various appropriate adjuvants are well known in the art as reviewed by Warren and Chedid, CRC Critical Reviews in Immunology 8:83 (1988), which is incorporated herein by reference. Some of the adjuvants which are currently

being tested include surface active agents, liposomes, immune-stimulating complexes, peptides, live attenuated vectors, enzymes such as galactose oxidase plus neuraminidase (NAGO), Bacille Calmette Guerin (BCG); Ribi Corp. DETOX formulation (Ribi DETOX); Complete and incomplete Freund's adjuvants, additionally containing Cell Wall Skeleton (CWS) and/or Monophosphoryl Lipid A (MPL) and Muramyl Dipeptide (MPD).

B. Examples

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other alternative procedures known to those skilled in the art may be alternatively employed.

EXAMPLE I

The preparation of the outer-envelope free viral particles is described in detail in copending U.S. patent application serial no. 200,752, incorporated herein by reference.

EXAMPLE II

Immunotherapy Of Seropositive Individuals

Nine individuals seropositive for HIV were treated by immunotherapy. An immunogen comprising non-infectious HIV particles devoid of the outer envelope proteins was prepared according to the method of Example I. The immunogen was emulsified in a 1 to 1 proportion in incomplete Freund's adjuvant (IFA) in an emulsifier (Spex 8000 Mixer Mill; Spex Industries Inc., Edison, N.J.). 1.0 ml of solution, containing 100 µg of protein, was administered intramuscularly. A booster of 100 µg of protein without adjuvant was administered 90 days later by intradermal injection.

The presence of HIV virus in a patient's peripheral blood lymphocytes was determined both pre- and post-immunization by cocultivation with freshly supplied peripheral blood lymphocytes stimulated with PHA and interleukin-2 (IL-2) by the method of Gallo et al., J. Clin. Microb. 25:1291 (1987), which is incorporated herein by reference. Kits to detect the presence of HIV antigens,

such as p24 are commercially available (for example, HIV p24 Assay; E. I. DuPont de Nemours & Co., Inc., Wilmington, DE). Each patient was tested 3 times prior to immunization, and at weeks 2, 4, 6, 8, 12 and 14 post immunization.

Table I provides the results of viral isolation from the patients. Patients 010 and 003, from both of whom HIV had been isolated prior to immunization, failed to exhibit isolatable virus through week 12 post immunization. Patients 008 and 009 were virus free by culture through week 8 post immunization. All four of these patients had exhibited high titers of anti-p24 (>1:5000) and RTI (>1:1000) before immunization. The remaining five patients, who exhibited lower anti-p24 and RTI titers prior to immunization, continued to exhibit isolatable virus after immunization.

TABLE I

IMMUNOLOGIC AND VIROLOGIC PROFILES IN SEROPOSITIVE VIREMIC ARC PATIENTS PRE- AND POST-IMMUNIZATION

	Cytologic		Immunologic			Virologic						
							Post (wks)					
Patient	T-4 Pre	T-4 Post	Anti-p24	RTI	N.A.	Pre	2	4	6	8	12	13
003	518	671	250,000	2,560	40	+--	-	-	-	-	-	
010	229	297	500,000	10,240	80	++-	-	-	-	-		
008	288	371	16,000	2,560	80	-+-	-	-	-	-	-	+
009	219	261	8,000	2,560	80	-+-	-	-	-	-	-	+
006	296	283	3,200	640	40	+-++	+	+	-	-	+	
001	237	198	3,200	20	40	+++	-	-	+	-	-	
004	261	277	400	<20	20	--+-	+	-	-	+	+	
007	180	259	200	80	10	+++	+	+	-	+	+	
005	228	98	170	<20	14	--++	+	+	+	+	+	

N.A. refers to neutralizing antibody.

EXAMPLE III

By varying the antigen dosage, the induction of immunity to retroviral infection in a human effective to induce a cell mediated immune response but subimmunogenic for a humoral immune response can be achieved.

The adjuvant IFA was utilized with varying doses and number of inoculations utilizing the non-infectious HIV antigen prepared above. Antigen doses of 50, 100, 200 and 400 µg were administered to 48 asymptomatic HIV-infected individuals having CD4 of 600 or greater and an HIV immunogen delayed type hypersensitivity (DTH) response of less than 5 mm induration. Serum IgG ranged from 600 - 3,000 mg/dl. The humoral (HIV antibody production) response rate was 7/16 (44%) in IFA controls, 3/8 (38%) in individuals receiving IFA + 50 µg HIV antigen, 6/8 (75%) in individuals receiving IFA + 100 µg HIV antigen, 7/8 (88%) in individuals receiving IFA + 200 µg HIV antigen, and 4/8 (50%) in individuals receiving IFA + 400 µg HIV antigen. However, only the 200 µg HIV antigen group was statistically significant when compared to the IFA control.

The induration size, as a measure of the strength of the DTH response, was compared over a 36 week period. The size of induration in the IFA control group varied from about 3 to about 5 mm during the study period, and was above the 3 mm size from week 12 to week 24. The size of induration in the group receiving 50 µg HIV antigen ranged from about 2 mm at week 4 to about 7.5 mm at week 16 and declined to the baseline level of about 3 mm by week 28. The size of induration in the group receiving 100 µg HIV antigen ranged from about 5 mm at week 4 to about 10 mm at week 12 and remained above about 5 mm for the duration of the study. The size of induration in the group receiving 200 µg HIV antigen ranged from about 4 mm at week 4 to about 9 mm at week 8 and slowly declined to reach about 4 mm by week 28. The size of induration in the group receiving 400 µg HIV antigen reached the maximum size of about 8 mm at week 4 and remained at about 7-8 mm for the duration of the study.

The cell mediated immune response rate was 7/16 (44%) in IFA controls, 6/8 (75%) in individuals receiving IFA + 50 µg HIV antigen, 7/8 (88%) in individuals receiving IFA + 100 µg HIV antigen, 7/8 (88%) in individuals receiving IFA + 200 µg HIV antigen, and 7/8 (88%) in individuals receiving IFA +

400 µg HIV antigen. The CMI response rate in all groups above 50 µg HIV antigen group was statistically significant when compared to the IFA control.

Thus, when dosage of antigen alone is considered, and only a single dose is administered, dosages of less than 200 µg HIV antigen achieved the desired effect of favoring development of a predominantly CMI response while being less likely to provoke a humoral response.

However, administration of lower dosages of antigen in more than one dosages could achieve a similar result. Additionally, one may vary the adjuvant in order to utilize an adjuvant which favors induction of a CMI response rather than a humoral response. As demonstrated in Example IV below, adjuvant choice can also have a great effect on the induction of a CMI rather than a humoral response.

EXAMPLE IV

Differences in antibody response and Cell Mediated Immunity (CMI) in guinea pigs after a single intramuscular injection of HIV-I Immunogen and each of three different adjuvants.

Female Hartley guinea pigs, approx. 90 days old at Day 0 were administered the test immunogen by an intramuscular injection on Day 0. The test immunogen comprised HIV-1 Immunogen in one of three Adjuvants. HIV-1 immunogen is an envelope depleted, gamma irradiated, beta-propiolactone treated HIV-1 virus preparation. Incomplete Freund's Adjuvant (IFA) consisted of 90% Drakeol 6VR mineral oil plus 10% Arlacel A. DETOX is a oil/water emulsion obtained from Ribi Corporation. The HIV-1 Immunogen in DETOX was prepared by adding 200 µg HIV-1 Immunogen to lyophilized DETOX containing 500 µg Cell Wall Skeleton (CWS) and 50 µg Monophospholipid A (MPL); sufficient saline was then added to bring the volume to 2.0 ml. The solution was mixed by drawing several times through a 21 g needle. The HIV-1 Immunogen in IFA alone was prepared by adding 1000 µg of HIV-1 Immunogen and sufficient saline to bring the volume to 5.0 ml. 5.0 ml of IFA was then added and emulsification was performed by mixing on a SPEX-5100 for 20 min. at room temperature. The HIV-1 Immunogen in CWS/MPL +IFA was prepared by adding 1000 µg of HIV-1 Immunogen and sufficient saline to bring the volume to 5.0 ml. 5.0 ml of 500 µg CWS/ml + 50 µg MPL/ml in IFA was then added to

bring the total volume to 10.0 ml. Emulsification was performed by mixing on a SPEX-5100 for 20 min. at room temperature.

The animals were divided into 3 groups. Group 1 received 100 µg HIV-1 Immunogen in 50% IFA alone. Group 2 received 100 µg HIV-1 Immunogen + 250 µg CWS + 25 µg MPL in 1.0 ml of 50% IFA. Group 3 received 100 µg HIV-1 Immunogen + 250 µg CWS + 25 µg MPL in 1.0 ml of 50% DETOX adjuvant formulation.

Animals were dosed with 1.0 ml of the test substance intramuscularly in the left thigh on Day 0. On Day 28, all animals were skin tested for Delayed Type Hypersensitivity (DTC) to HIV with 10.0, 1.0, 0.1 and 0 µg of HIV-1 Immunogen in saline. Measurements for erythema and induration at the skin test site were taken 24 and 48 hours after the skin test administration. After the 48 hour skin test reading, the animals were euthanized in a CO_2 chamber and bled by cardiac puncture. Serum was assayed by Enzyme Linked ImmunoSorbent Assay (ELISA) for antibody to p24 and by Western Blot for total anti-HIV serological response, Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque and plated at 2 X 10^5/well for the antigen proliferation assay. The proliferative response to the HIV-1 Immunogen (5.0 µg/ml), tetanus toxoid (1:200), and phytohemagglutinin (PHA) (10 µg/ml) were measured to assess cell mediated immunity to HIV-1 Immunogen with the tetanus and PHA included as negative and positive controls respectively.

Antibody titers, Western Blot scores and Skin Test scores of each test group were averaged by geometric mean. Antigen Proliferation data were calculated on a percent responding basis. Each of these assays is described in more detail below.

A. <u>Delayed Type Hypersensitivity (DTH)</u>

Prior to skin test administration, the hair was removed from the right flank of each animal with an electric clipper. A depilatory was then applied for 20 minutes and removed with a wet sponge. One injection of each of four concentrations (10.0, 1.0, 0.1, and 0 µg of HIV-1 Immunogen in 0.1 ml of saline was then injected just below the first layer of skin (I.D.). Erythema and induration were measured in millimeters with a caliper at 24 and 48 hours after

injection. Skin test scores were calculated by subtracting the 0 µg (saline only) measurement from the other measurements of the same animal at the same time point.

All HIV skin tested animals showed skin test reactivity at 24 and 48 hours. In the IFA group, the reactivity peaked at 24 hours and showed slightly less reactivity at 48 hours. Total reactivity in the CWS/MPL + IFA group was equal at 24 and 48 hours, while total reactivity in the DETOX group was lower at 24 than 48 hours. Figures 1 and 2 show the induration scores for the 10 µg HIV skin test for each animal.

The mean scores of the three groups (Figure 1) show that the CWS/MPL + IFA group exhibited the greatest skin test reactivity. The DETOX group was slightly more potent than the IFA group in inducing this response.

B. Antigen Proliferation

Peripheral blood lymphocytes (PBL) were separated by Ficoll-Hypaque and plated in a 96 well plate at $2X10^5$/ml. Proliferation in response to HIV-I Immunogen was assayed by the addition of HIV-I Immunogen to the wells at 5.0 µg/ml. Tetanus and PHA were assayed at 1:200 dilution of stock material and 10 µg/ml, respectively. Cells were incubated for 1 week with or without antigen and labeled on the sixth day with ^{3}H-Methionine overnight. Cells were harvested on the seventh day and assayed for incorporated label. Data was represented as the Stimulation Index (SI). All assays were done in triplicate and the numbers averaged. If the higher and lower number for each assay were at least two fold different than the middle number, the data was considered inconclusive and was not reported. An SI of >1.95 was considered to be a positive response.

SI = cpm of wells with antigen /cpm of wells without antigen.

No animals responded to tetanus which was included as a negative control. One animal from the IFA group and one animal from the CWS/MPL + IFA group did not respond to the positive control (PHA) and were not included in the data analysis. Antigen specific proliferation to the HIV Immunogen was detected in 100% of the CWS/MPL + IFA group, 40% of the IFA group and 100% of the DETOX group (See Figure 3).

C. ELISA

Antibody to p24 was determined by ELISA assay on 96 well HIV r-p24 coated microtiter plates. The coated plates were blocked with BSA diluent and incubated with diluted test animal serum samples (subsequent two fold dilutions were run in order to determine an endpoint dilution). After washing each well three times with wash buffer, the plates were then incubated with Horseradish Peroxidase-conjugated Goat Anti-Guinea Pig IgG. The plates were washed again and then incubated with a substrate solution which causes a color change in relation to the amount of HIV specific antibody detected. The plates were read on an Intermed Immuno-reader NJ-2000 at 405 nm to determine the optical density of each well. The reported endpoint titer was the dilution between 0.05 and 2.00 and closest to 0.1 optical density units (O.D.) after the reagent control O.D. has been subtracted. If results were outside the range of assay limits, "< or >" were used. The titer was not entered into statistical analysis unless it was confirmed.

HIV immunogen plus CWS/MPL + IFA and IFA immunized animals demonstrated a measurable antibody response to p24 by ELISA. No detectable response to p24 was measured in the HIV immunogen plus DETOX immunized animals. Figures 4 and 5 show the antibody titers for each animal. The mean antibody titer of the CWS/MPL + IFA group (Figure 4) was significantly higher than the IFA group.

D. Western Blot

Western Blotting is a method by which proteins separated by gel electrophoresis are transferred to a nitrocellulose sheet. HIV antibody present in the sample will bind to antigens located on the strip as discrete bands. Unbound material is aspirated, the strip washed and Horseradish Peroxidase conjugated goat anti-Guinea Pig IgG is added. The strip is then washed to remove unbound conjugate and a substrate is added. The presence of antibody is indicated by the appearance of purple bands at specific HIV viral positions on the strip from highest to lowest molecular weight. The serological responses elicited by the test adjuvant plus antigen were scored by counting the total number of protein bands on each strip.

HIV immunogen plus CWS/MPL + IFA and IFA immunized animals demonstrated a measurable antibody response to p24 by Western Blot. A detectable response to p24 was measured in only one of the HIV immunogen plus DETOX immunized animals. The scores (total protein bands for each animal are presented in Figures 6 and 7. The mean score (total number of bands/total number of animals) for the IFA group was slightly higher than the CWS/MPL + IFA group and dramatically higher than the mean score of the DETOX group.

Summary

When ranked according to antibody response to HIV by ELISA and Western Blot, the relative potencies of the adjuvant groups were as follows:

ELISA: CWS/MPL + IFA > IFA > DETOX
Western Blot: IFA > CWS/MPL + IFA > DETOX

When ranked according to Cell Mediated Immunity to HIV by DTH and antigen proliferation assays, the relative potencies of the adjuvant groups were as follows:

DTH: CWS/MPL + IFA > DETOX > IFA
Ag Proliferation: CWS/MPL + IFA = DETOX > IFA

All four assays clearly showed that a single dose of CWS/MPL + IFA at a concentration of 250 µg CWS + 25 µg MPL + 50% IFA was more effective in eliciting both an antibody and a CMI response to HIV in guinea pigs than IFA alone, while DETOX was less effective in eliciting an antibody response but equally as effective as CWS/MPL + IFA in eliciting a CMI response. Based on these results, the DETOX containing antigen composition is better suited to obtain the objective of the present invention to induce a CMI response while minimizing the antibody response.

EXAMPLE V

Determination of optimal dosage of antigen preparation and optimal adjuvant.

Six hundred Balb/C mice (8 weeks old) are divided into four groups and immunized with various dosages of HIV-immunogen ranging from about 1 pg to

about 10 µg which have been prepared in each of the following adjuvant formulations: Bacille Calmette Guerrain (BCG); Ribi Corp. DETOX formulation (Ribi DETOX); Cell Wall Skeleton plus Monophosphoryl Lipid A plus Incomplete Freund's Adjuvant (CWS/MPL/IFA) and Muramyl Dipeptide (MPD). Each group of 150 mice is immunized using one of the adjuvant formulations. Mice are immunized with 0, 1 pg, 100 pg, 10 ng, 1µg, or 10 µg of HIV-1 immunogen. Each immunogen dosage is tested using each of the adjuvant formulations.

Immunizations are administered on Day 0 as a 0.1 ml subcutaneous injection containing the appropriate amount of HIV-1 immunogen formulated in the appropriate adjuvant. The following immunological assays are used to determine the effects of immunogen dosage, immunization schedule, and adjuvant formulation on both cell mediated immunity and antibody responses.

Skin Test: HIV-1 immunogen in saline is given by footpad injection. Swelling is measured by calipers at 6, 24, and 48 hours post-injection .

Anti-p24 antibody. A challenge dose containing 10 µg of HIV-I immunogen in IFA is administered intraperitoneally. Two weeks after the challenge dose, animals are sacrificed. The levels anti-p24 antibody are measured using a standard enzyme-linked ImmunoSorbent Assay (ELISA) test.

Cytokine levels. After the animals are sacrificed, the levels of gamma-interferon and Interleukin-4 are also measured, using commercially available kits obtained from Endogen Corp. (Boston, Mass.). Methods to measure the cytokine levels are known to those of skill in the art and described in Salgame, et al.; 1991. *Science* 254:279 and Street, et al.; 1991. *FASEB J.* 5:171.

For these assays, the animals from each Immunization Group are divided into 5 subgroups (each subgroup containing 5 mice). The subgroups are boosted and assayed using the following schedule. All times are expressed with respect to the initial immunization given on Day 0.

The animals of Group A are assayed at 1 month. The animals of Group B are assayed at 2 months. The animals of Group C are assayed at 3 months.

The animals of Group D are administered a booster immunization at 1 month and assayed at 3 months. The animals of Group E were administered a booster immunization at 1 month and 2 months and assayed at 3 months.

The immunization protocol which provides maximal CMI with minimal humoral response is determined for practicing the present invention.

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WE CLAIM:

1. A method of inducing immunity to retroviral infection in a human or other mammal comprising administration of a preparation comprising retroviral antigen in an amount effective to induce a cell mediated immune response substantially free of a humoral immune response.

2. The method of claim 1 wherein said antigen comprises non-infectious retroviral particles devoid of outer envelope proteins.

3. The method of claim 2 wherein said retroviral infection is an HIV infection and said retroviral particles are HIV particles.

4. The method of claim 2 wherein said outer envelope proteins are gp160 and gp120.

5. The immunogen of claim 1 wherein said retroviral particles are rendered non-infectious by treatment with gamma radiation and beta-propiolactone.

6. The method of claim 1 wherein said antigen preparation comprises one or more retroviral proteins, with the provision that none of said retroviral proteins is an outer envelope protein.

7. The method of claim 1 wherein said antigen preparation comprises one or more HIV proteins, with the proviso that none of said HIV proteins is an outer envelope protein.

8. The method of claim 1 wherein said antigen preparation additionally comprises an adjuvant.

9 The method of claim 7 wherein said adjuvant is DETOX.

10. A method for inducing an immunologically protective state in humans or animals who are seronegative for HIV comprising: administering to an individual who is seronegative for HIV and who is also asymptomatic for AIDS HIV antigens incorporated in an immunological adjuvant in an amount effective to induce CMI substantially free of a humoral immune response .

11. The method of claim 9 wherein said antigen is administered at a dose of less than 200 μg.

FIGURE 1

SKIN TEST - 10 ug HIV INDURATION

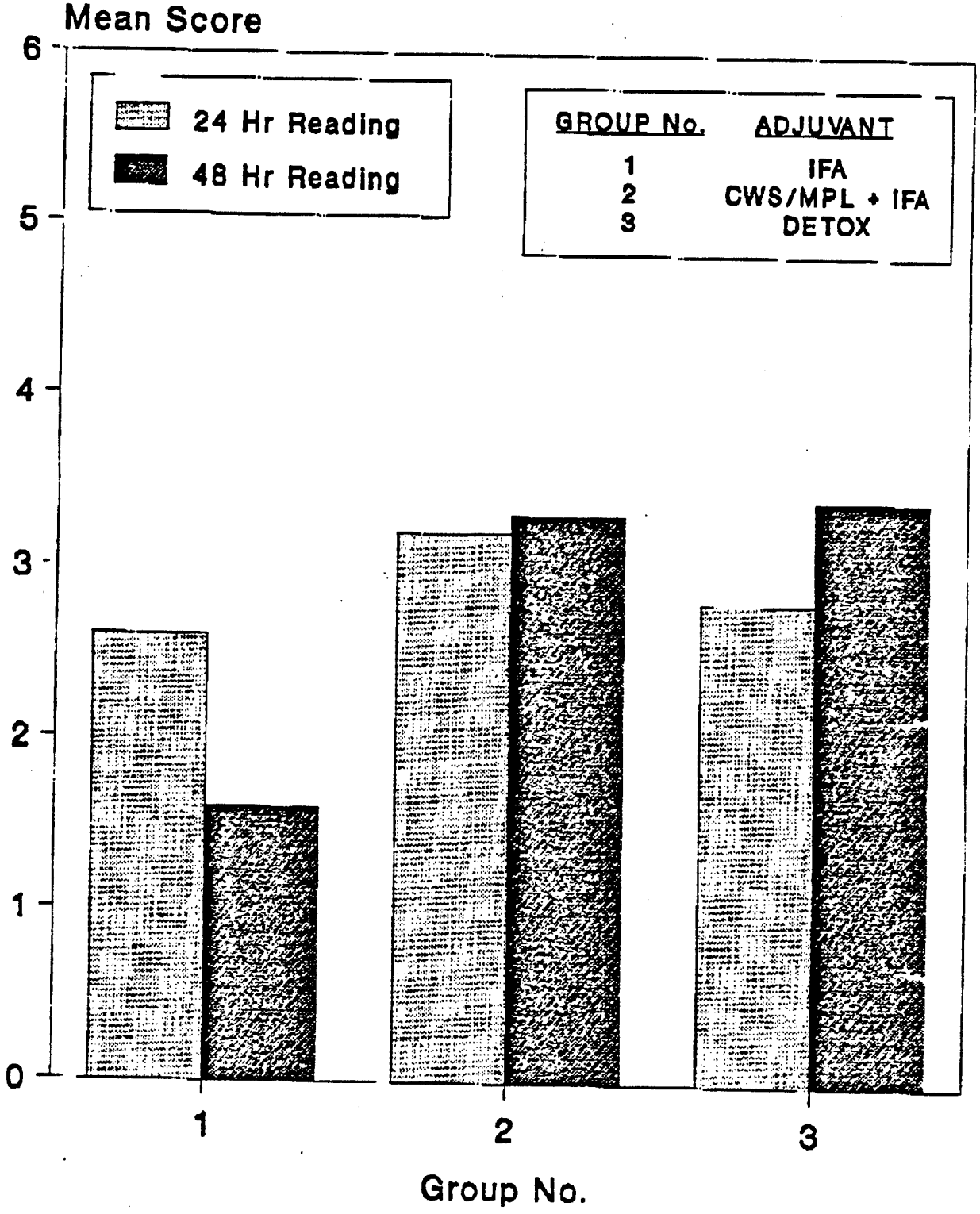

FIGURE 2

SKIN TEST - 10 ug HIV INDURATION

Group No.	Test Material	Animal No.	Induration Score		Group Mean	
			24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	50% IFA + HIV-1 IMMUNOGEN	19	3	2		
		20	2	1		
		21	DEAD	DEAD		
		22	3	2		
		23	3	1		
		24	2	2	2.6	1.6
2	CWS/MPL +IFA + HIV-1 IMMUNOGEN	25	5	4		
		26	4	1		
		27	3	5		
		28	2	3		
		29	4	4		
		30	1	3	3.2	3.3
3	DETOX + HIV-1 IMMUNOGEN	31	3	4		
		32	3	4		
		33	4	4		
		34	1	DEAD		
		35	3	2		
		36	3	3	2.8	3.4

FIGURE 3

ANTIGEN PROLIFERATION ASSAY FOR CELL MEDIATED IMMUNITY (CMI)

Group No.	Test Material	Animal No.	HIV	TETANUS (Neg. Control)	PHA * (Pos. Control)	% Response
1	50% IFA + HIV-1 IMMUNOGEN	19	-	-	-	
		20	-	-	+	
		21	DEAD			
		22	+	-	+	
		23	-	-	+	
		24	+	-	+	50 %
2	CWS/MPL + IFA + HIV-1 IMMUNOGEN	25	+	-	+	
		26	+	-	-	
		27	+	-	+	
		28	+	-	-	
		29	+	-	+	
		30	+	-	+	100 %
3	DETOX + HIV-1 IMMUNOGEN	31	+	-	+	
		32	+	-	+	
		33	+	-	+	
		34	DEAD			
		35	+	-	+	
		36	+	-	+	100 %

* - Any animal not responding to PHA was not included in the data analysis for this assay.

FIGURE 4

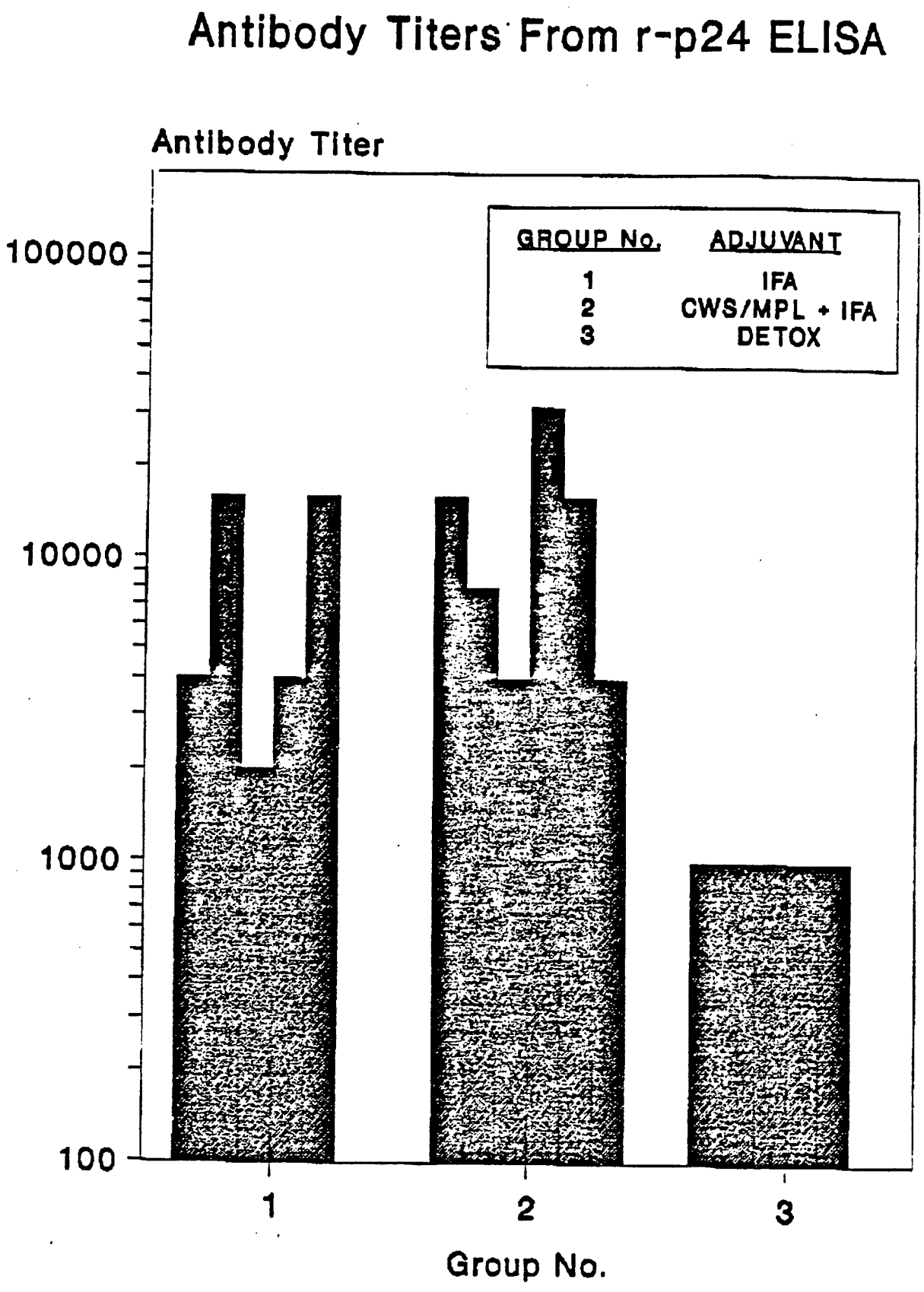
Antibody Titers From r-p24 ELISA
Antibody Titer
100000
10000
1000
100
GROUP No. ADJUVANT
1 IFA
2 CWS/MPL + IFA
3 DETOX
1
2
3
Group No.

FIGURE 5

ANTIBODY TITERS FROM r-p24 ELISA

Group No.	Test Material	Animal No.	Endpoint Titer	Group Mean
1	50% IFA + HIV-1 IMMUNOGEN	19	4000	
		20	16000	
		21	DEAD	
		22	2000	
		23	4000	
		24	16000	8400
2	CWS/MPL + IFA + HIV-1 IMMUNOGEN	25	16000	
		26	8000	
		27	4000	
		28	32000	
		29	16000	
		30	4000	13333
3	DETOX + HIV-1 IMMUNOGEN	31	<1000	
		32	<1000	
		33	<1000	
		34	DEAD	
		35	<1000	
		36	<1000	<1000

FIGURE 6

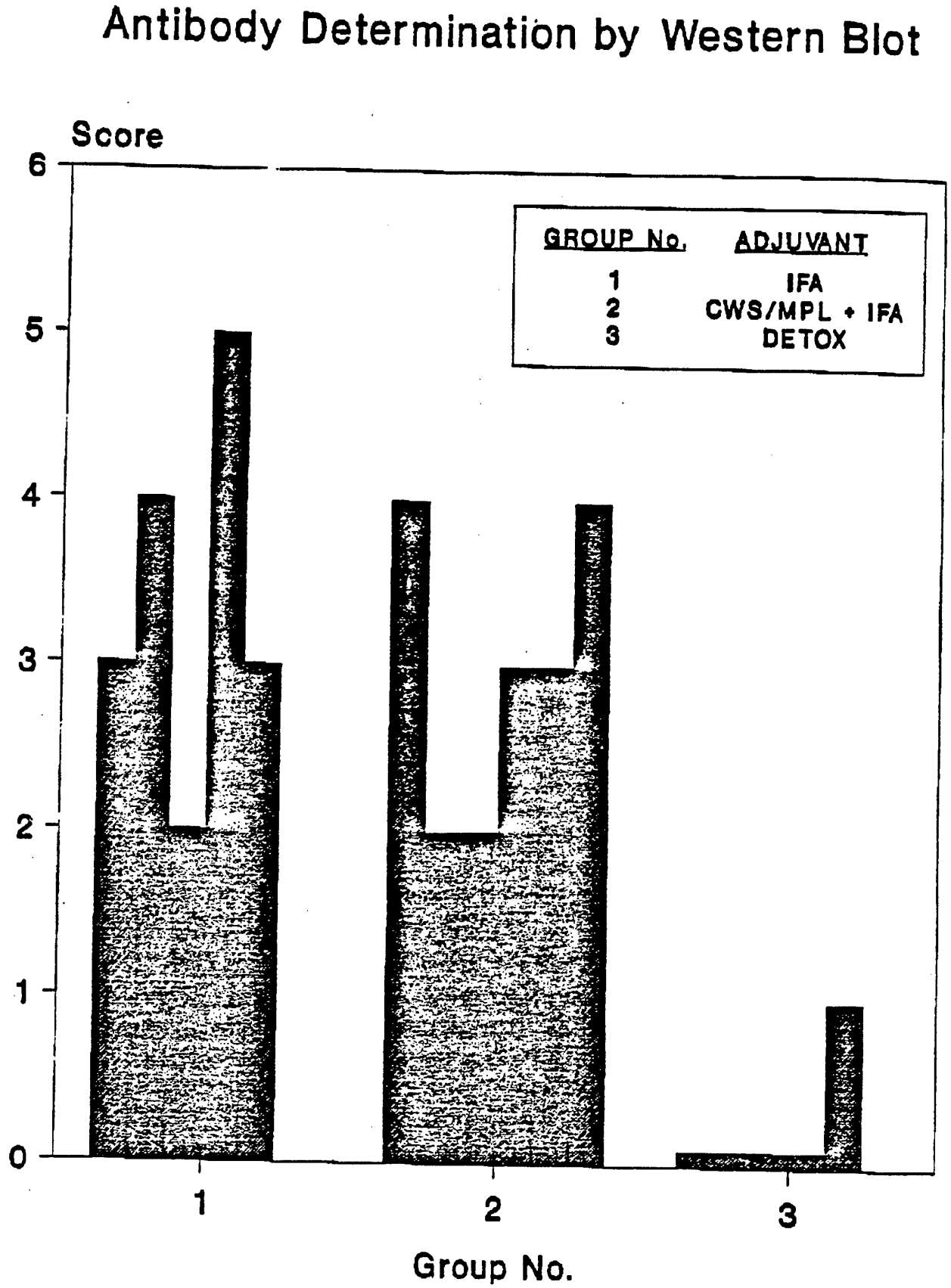
Antibody Determination by Western Blot
Score
6
5
4
3
2
1
0
GROUP No.
ADJUVANT
1
IFA
2
CWS/MPL + IFA
3
DETOX
1
2
3
Group No.

FIGURE 7

WESTERN BLOT SCORES

Group No.	Test Material	Animal No.	Score	Group Mean
1	50% IFA + HIV-1 IMMUNOGEN	19	3	
		20	4	
		21	DEAD	
		22	2	
		23	5	
		24	3	3.4
2	CWS/MPL + IFA + HIV-1 IMMUNOGEN	25	4	
		26	2	
		27	2	
		28	3	
		29	3	
		30	4	3.0
3	DETOX + HIV-1 IMMUNOGEN	31	0	
		32	0	
		33	0	
		34	DEAD	
		35	0	
		36	1	0.2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06820

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/00
US CL :424/89

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/89

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

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

APS, Dialog
search terms: HIV, vaccine, non-infectious, devoid, outer envelope, adjuvant, detox

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, A, 2,500,440 (Salk et al) 15 December 1988, Abstract, see entire document.	1-11
Y	WO, A, 8,809,670 (Immune Response) 15 December 1988, abstract, see entire document.	1-11
Y	Science, Volume 256, issued 12 June 1992, Zheng et al, "Galactose Oxidation in the Design of Immunogenic Vaccines", pages 1560-1563, see entire article.	9-11
Y	AIDS Vaccine:Research and Clinical Trial, issued 1990, Salk et al, "Prophylactic and Therapeutic Immunization Against AIDS", pages 265-275, see entire article.	1-11

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

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be part of particular relevance	
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"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	

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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHRISTINE M. NUCKER Telephone No. (703) 308-0196

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