METHODS FOR TREATMENT OF DISEASES ASSOCIATED WITH A DEFICIENCY OF FAS LIGAND ACTIVITY

Method for treating a patient with a condition characterized by a deficiency of Fas ligand activity comprising the step of administering to the patient an agent that increases the Fas ligand activity in the patient.
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DESCRIPTION

METHODS FOR TREATMENT OF DISEASES ASSOCIATED WITH A DEFICIENCY OF FAS LIGAND ACTIVITY

Field of the Invention

This invention relates to methods for the treatment and monitoring of diseases associated with a deficiency of Fas ligand activity.

Background of the Invention

The following is a general discussion of relevant art, none of which is admitted to be prior art to the invention.

Human immunodeficiency virus disease is a major health problem throughout the world. The hallmark of HIV disease is the gradual, inexorable loss of CD4+ T lymphocytes. The loss of these T-cells is an important pathogenetic aspect for patients infected with HIV. Maintaining a patient's T-cell population is the goal of current HIV prevention and treatment protocols.

Recent studies have shown that the production of virus by infected individuals is an important variable in the progression of the disease (Wei, X. et al., Nature,

Lu et al., *J. Virol.*, 68:390, 1994, disclose that anti-Fas monoclonal antibodies kill chronically infected cells of a CD4+ monocytoid tumor cell line expressing the HIV protein gp160. Biswas et al. disclose that anti-Fas monoclonal antibodies can kill cells chronically infected with HIV and decrease the number of infected cells in vitro and thereby decreases viral replication (Biswa et
al., J. Virol., 68:2598, 1994). Kobayashi et al. disclose that in vitro anti-Fas monoclonal antibody selectively kills chronically HIV-infected tumor cells. Kobayashi et al. state that "[p]erhaps anti-Fas mAb may also be clinically applicable in HIV infection but further studies are required to test this possibility". (Kobayashi, N. et al., Proc. Natl. Acad. Sci. USA, 87:9620, 9623-9624, 1990).

Ogasawara et al. disclose that intraperitoneal administration of anti-Fas antibodies into mice resulted in rapid killing due to severe damage of the liver by apoptosis. They state "...possible therapeutic uses of the agonistic antibodies against the Fas antigen were proposed for the treatment of EBV-induced lymphoproliferative lesions in immunocompromised individuals, HTLV-1 associated malignant disorders or AIDS patients. Such approaches must be carefully controlled in the light of our present results". (Ogasawara et al., Nature, 364:806, 1993).


Analysis of T-cell death has indicated that a major pathway for apoptosis of these cells involves the interac-


Banda et al., supra, disclose that the HIV protein gp120 primes cells for activation induced cell death and suggest that this may be a mechanism for CD4+ T cell population depletion in acquired immune deficiency syndrome (AIDS).

Groux et al. supra, disclose that lymphocytes from HIV infected patients were more susceptible to cell death in vitro after stimulation with pokeweed mitogen or staphylococcal enterotoxin B, but not after stimulation with phytohemagglutinin. They suggest "that activation-induced T cell death might also occur in vivo and account for the progressive depletion of CD4+ T cells that leads to AIDS".
Meyaard et al., *supra*, disclose that deletion of antigen reactive T-cells in HIV-1 infected individuals is enhanced and suggest that programmed cell death may be responsible for deletion of these T-cells and this may contribute to HIV-induced immunodeficiency.

Debatin et al., *Blood*, 83:3101, 1994, disclose that in both CD4⁺ and CD8⁺ T-cells from HIV infected patients, the level of Fas (APO-1) is increased indicating a potential link between APO-1-mediated apoptosis and T-cell depletion in AIDS.

Oyaizu et al., *Blood*, 84:2622, 1994, demonstrate that crosslinking of CD4 molecules on T-cells by anti-CD4 monoclonal antibodies or HIV GP 160 upregulates the expression of Fas antigen on those cells and may play a critical role in bringing about peripheral T-cell apoptosis and contribute to HIV disease pathogenesis.

Westendorp et al., *Nature*, 375:497, 1995, demonstrate that HIV-1 Tat protein gp120 sensitize CD4⁺ T-cells to apoptosis by upregulation of Fas antigen (CD95) and conclude that reduction in "increased pathological CD95-mediated apoptosis to physiological levels may allow new therapeutic interventions in AIDS".

Katsikis et al., *J. Exp. Med.*, 181:2029, 1995, disclose that peripheral blood CD4⁺ and CD8⁺ T-lymphocytes from infected individuals undergo apoptosis *in vitro* in response to antibody stimulation of Fas and at a higher frequency than uninfected controls and conclude "that CD4⁺ and CD8⁺ T lymphocytes in HIV-infected individuals are primed in vivo to undergo apoptosis in response to Fas
stimulation, suggesting that Fas signaling may be responsible for the T lymphocyte functional defects and depletion observed in HIV disease.

Summary of the Invention

Applicant's invention is based on the novel observation that Fas antigen mediated killing is attenuated in certain diseases such as HIV infection. The current invention is based on the replacement of Fas ligand activity to levels greater than what is observed in the diseased state. The replacement of Fas ligand activity allows for increased apoptosis to occur.

Fas ligand is known to play an important role in the physiological cell death of T cells. In gld/gld mice that do not express Fas ligand activity, the immune system and in particular the T lymphocytes, function inappropriately and ineffectively (Ramsdell et al., Eur. J. Immunol., 24:928, 1992; Takahashi et al., Cell 76:969, 1994). Depression of Fas ligand activity, as observed in HIV disease, disables the physiological processes of T cell turnover. Therapy with Fas antigen agonists will benefit patients by restoring physiological T cell turnover.

Other molecules have been shown to participate in the pathway that results in apoptosis. IL-10 contributes to an inhibition of T-cell proliferation while protecting T-cells from accelerated apoptosis. (Taga et al., Int. Immunol., 5:1599-1608, 1993; Taga et al., J. Clin. Invest., 94:251-260, 1994; Taga et al., J. Immunol., 148:1143-1148, 1992). Bcl-2 has been shown to antagonize the

Applicant has also discovered that Herpes Simplex virus type 2 (HSV2) inhibits Fas ligand activity. HSV2 infected patients could also be treated with Fas ligand or Fas antigen agonists or other agents encompassed by the claimed invention so as to increase Fas ligand activity. Other members of the Herpes virus family, including Human herpes virus 6 (HHV6), Epstein-Barr virus (HHV4) and cytomegalovirus may also inhibit Fas ligand activity. The increase of Fas ligand activity might have broad application in a variety of herpes viral infections.

Other diseases that involve a deficiency of Fas ligand activity can be determined by comparing the level of Fas ligand activity or the amount of Fas ligand expressed on peripheral blood cells, such as monocytes, in a patient and in a healthy individual. These diseases are also candidates for therapy that increases Fas ligand activity as described in the current invention.
The invention also encompasses various methods to specifically target cells to which it is advantageous to bring about natural cell death (apoptosis) and thus avoid exposure of cells expressing the Fas antigen which are not intended as targets for apoptosis. These procedures minimize toxic effects produced by systemic administration of molecules such as anti-Fas antigen antibodies. Such methods include attaching the Fas ligand or Fas antigen agonist to an inert substance (e.g., a biodegradable bead) to which is also affixed a targeting molecule for a specific cell type. Targeting molecules include cytokines, antibodies, and other ligands for cell surface molecules. T-cells can be targeted for apoptosis by utilizing antibodies with specificity for CD3, CD4, CD8, CD5, CD7, or CD2 which are molecules expressed on the surface of T lymphocytes. Another method to target specific cells with Fas ligand activity is to utilize bispecific antibodies directed to both Fas antigen and a cell surface molecule on the target cell. Another method useful to avoid systemic toxicity is to remove cells from a patient, treat the cells ex vivo and reintroduce cells back into the patient. In addition, by monitoring the Fas ligand activity in the patient during the course of treatment the dose of therapeutic reagent can be optimized, thus avoiding the potential problem of systemic toxicity.

The invention also includes a method for determining when to initiate therapy with Fas antigen agonists by determining whether there is a deficiency in Fas ligand
activity. Early loss of Fas ligand activity provides a useful indicator for the initiation of drug therapy. A deficiency in Fas ligand activity can also be ascertained by determining the amount of Fas ligand on peripheral blood cells (e.g., monocytes) or in serum, or by determining the amount of Fas antigen on peripheral blood cells.

In addition, the invention includes methods for monitoring Fas ligand activity during the course of treatment. Monitoring includes determining the level of Fas ligand activity, determining the amount of Fas ligand on peripheral blood cells or in serum, or determining the amount of Fas antigen on peripheral blood cells. The amount of Fas antigen expressing cells will decrease with the increase in Fas ligand activity.

Thus, in a first aspect, the present invention features a method for treating a patient with a condition characterized by a deficiency of Fas ligand activity. The method comprises the step of administering to the patient an agent which increases the Fas ligand activity in the patient.

A patient with a condition characterized by a deficiency of Fas ligand activity can be identified by comparing the level of Fas ligand activity, or Fas ligand in peripheral blood cells or serum, or Fas antigen in peripheral blood cells of a patient with those of a normal individual (i.e. not suffering from a disease condition).

By "administering to the patient" is meant injecting or placing into an infected person or animal or applying
on the skin of the infected person or animal or infusing cells after prior removal and ex vivo treatment.

By "agent which increases Fas ligand activity" is meant Fas ligand or portion thereof or another agonist of Fas antigen that elevates the level of Fas ligand activity.

By "Fas ligand activity" is meant the ability to initiation apoptosis.

By "targeted cell" is meant a cell in which apoptosis is to be initiated.

In a second aspect, the present invention features a method for treating viral infection in a patient. The method comprises the step of administering to the patient an agent that increases apoptosis of Fas' cells in the patient.

By "agent" is meant an agonist for the process of apoptosis. Examples of agonists include the Fas ligand or portion thereof, or other agonists of the Fas antigen, such as an anti-Fas antibody.

By "apoptosis" is meant programmed cell death in response to a variety of different triggers.

By "increase" is meant elevation above the level observed in affected individuals.

By "Fas" is meant the Fas antigen (CD95) which is a cell surface molecule which plays a role in the induction of apoptosis in cells by binding the Fas ligand.

In preferred embodiments, the viral infection is caused by human immunodeficiency virus; the viral infection is caused by herpes simplex virus type 2. In other
preferred embodiments for both the first and second aspect of the invention, the agent comprises a portion of the Fas ligand or another agonist sufficient to induce or allow apoptosis; the agent comprises an anti-Fas antibody; the anti-Fas antibody is humanized; the agent comprises an anti-Fas antibody linked to a toxin; the agent comprises a bispecific antibody including domains with specificity for Fas antigen and for a cell surface marker specific for infected or targeted cells; the agent includes a targeting molecule; the agent comprises a Fas antigen agonist and a first associating domain and a targeting molecule and a second associating domain; the Fas ligand or agonist is provided by protein transfer to peripheral blood cells; the Fas ligand is provided by gene expression of Fas ligand to peripheral blood cells.

By "human immunodeficiency virus" is meant a retrovirus which causes an infection in humans or other primates associated with the eventual development of immunodeficiency.

By "herpes simplex virus type 2" is meant a herpesvirus which causes an infection in humans characterized by cutaneous ulcerations and persistence in nervous tissue.

By "portion of Fas ligand" is meant the entire Fas ligand (amino acid residues 1-281) or the extracellular portion of Fas ligand (amino acid residues 103-281) (Takahashi et al., Int. Immunol., 6:1567 1994), incorporated herein by reference. The present invention can utilize a wide range of alterations of Fas ligand, e.g. substitutions and deletions, that keep intact the active
portion of the molecule. Active domains or essential components can be determined by mutagenesis procedures well known in the art. Portions of Fas ligand can be truncated or mutated and then tested to see if the altered molecule retains its ability to initiate apoptosis through its interaction with the Fas antigen.

By "Fas agonist" is meant a molecule or substance that activates or binds Fas antigen on a cell and initiates apoptosis of that cell.

By "trigger" is meant that the agent activates or binds Fas antigen so as to result in the initiation of apoptosis.

By "anti-Fas antibody" is meant an antibody that activates or binds to the Fas antigen on a cell and results in apoptosis of that cell.

By "humanized" is meant an antibody of a species other than humans that has the constant regions and the framework domains of the variable regions of the molecule substituted with human sequences while maintaining the antigen binding domains from the original nonhuman antibody. Techniques for the construction of humanized antibodies are known to those who practice the art. (Tempest et al., Protein Engineering, 7:1501, 1994; Benharet et al, Proc. Natl. Acad. Sci. USA, 91:12051, 1994).

By "anti-Fas antibody linked to a toxin" is meant an antibody directed toward the Fas antigen which is linked to a molecule able to bring about the killing of a cell.
Examples of such toxin molecules include pseudomonas exotoxin and diphtheria toxin.

By "bispecific antibody" is meant an antibody that includes component chains that bind to 2 different antigens. Methods of construction of bispecific antibodies are known to those of ordinary skill in the art and can be practiced as disclosed by Kostelny et al., J. Immunol., 148:1547, 1992.

By "targeting molecule" is meant a molecule that does not by itself possess the capacity to bind to Fas antigen but instead binds to a specific molecule that is expressed on the cells to be targeted for apoptosis. A targeting molecule can be linked to a Fas ligand or another Fas antigen agonist directly or via an inert substance such as a biodegradable bead.

By "first associating domain" is meant a sequence such as a fos leucine zipper sequence that can associate with a "second associating domain" on a separate molecule, such as a jun leucine zipper sequence. Chimeric molecules such as a Fas antigen agonist and a first associating domain and a targeting molecule and a second associating domain can be constructed via routine genetic engineering methods known by those who practice the art as in Kostelny et al., supra. Association of the Fas antigen agonist and a first associating domain and a targeting molecule and a second associating domain can occur on the cell surface of the targeted cell. Association promotes aggregation of Fas antigen which may be necessary for the signal transduction required to trigger apoptosis. Cell surface association
allows for the level of apoptosis to be more finely con-
trolled. In addition, the use of targeting molecules with
associating domains also confers specificity to the
initiation of apoptosis as the associating event only
occurs on cells actually targeted for apoptosis.

By "protein transfer" is meant a process by which the
external surface of a cell membrane is contacted with an
agent such that the agent is incorporated into the mem-
brane and is available for interaction with molecules via
an extracellular route.

By "peripheral blood cells" is meant any cell that is
present in a blood of a patient, e.g. lymphocytes, mono-
cytes, and leukocytes.

By "gene expression" is meant that a nucleic acid
encoding the Fas ligand or portion thereof or another Fas
antigen agonist is transfected into peripheral blood cells
and is expressed as protein on the surface of cells.

In a third aspect, the present invention features
methods for determining when to initiate Fas ligand
replacement therapy by determining the Fas ligand activity
level of peripheral blood cells of a patient, such as one
infected with HIV, or determining the amount of Fas ligand
on peripheral blood cells or in the serum of a patient, or
determining the amount of Fas antigen on peripheral blood
cells in a patient.

By "Fas ligand replacement therapy" is meant the
elevation or increase of levels of Fas ligand activity in
the peripheral blood of patients.
By "Fas ligand activity" is meant the ability of the Fas ligand to bring about killing of target cells by interacting with Fas antigen on those cells. Target cells useful for assessing Fas ligand activity include Jurkat cell, Suptl3 cells or Fas antigen transfected cells. Assays to monitor Fas ligand activity include cytotoxicity Jam assays and other assays familiar to those who practice the art.

In a fourth aspect the invention features methods to monitor Fas ligand activity replacement therapy by either determining Fas ligand activity of peripheral blood cells of a patient, determining the amount of Fas ligand on peripheral blood cells or in the serum of a patient, or determining the amount of Fas antigen on peripheral blood cells in a patient.

The amount of Fas ligand can be determined by any method that measures Fas ligand protein. In a preferred embodiment, the amount of Fas ligand is determined by antibody staining. Antibody staining involves the specific binding of a labelled antibody (e.g., fluoresceinated antibody) to cells with Fas ligand on its surface and detecting binding by measuring the label, e.g. via flow cytometry. Fas ligand activity can also be determined by an ELISA assay (Jones et al., Pediatr. Allergy Immunol., 5:230, 1994; Mariotti et al., J. Endocrinol. Invest., 17:861, 1994) for soluble Fas ligand (Tanaka et al., The EMBO Journal, 14:1129, 1995) present in the patients serum. These techniques are known to those who practice the art.
The amount of Fas antigen can be determined with specific antibodies for Fas antigen and flow cytometric analysis of peripheral blood mononuclear cells. These techniques are known to those who practice the art.

The current invention offers at least three major advantages compared to standard methods of treatment of HIV disease. Therapies directed at decreasing the production of virus in infections such as HIV, have typically utilized drugs directed at specific viral components (protease and reverse transcriptase) but thus far the capacity of HIV to mutate has limited the usefulness of these drugs. The proposed invention circumvents this problem as a specific viral protein is not the target of the therapy. Rapid mutation of the virus so as to circumvent this therapy is not likely.

The current method also involves a naturally occurring process in patients as opposed to treatment with a non-natural drug which may produce undesirable side effects. In addition, the invention allows for targeting specific cells responsible for the disease condition.

The invention also includes methods to monitor the increase of Fas ligand activity so that potential toxicity can be avoided. By accurate assessment of Fas ligand activity in a patient treatment can be tailored to the individual patient and the disease condition in that patient.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment and from the claims.
Brief Description of the Figures

Figure 1A is a graph comparing Fas ligand activity in cells from HIV infected and healthy patients. Closed symbols indicate the killing in the presence of the isotype control antibody (IgG1) and open symbols indicate the killing in the presence of the Fas ligand/Fas inhibitory antibody. The difference between the two curves is a measure of Fas ligand activity. The percent of specific killing is expressed on the y-axis. The effector cell to target cell ratio is expressed on the x-axis.

Figure 1B is a graph illustrating percentage of ligand dependent Fas ligand dependent killing in healthy donors and HIV patients. Percent Fas ligand dependent killing was determined by subtracting the percent of background killing on anti-Fas treated target cells from the percent of killing against isotype control treated target cells at an E:T of 20:1. Average Fas dependent killing is indicated by the horizontal line. A two-tailed T test was performed which indicated statistical significance, p<0.001. The percent Fas ligand dependent specific killing is expressed on the y-axis. The source of the effector peripheral blood mononuclear cell is indicated on the x-axis.

Figure 2 is a series of two-dimensional histograms indicating the expression of Fas antigen on T lymphocytes from healthy and HIV infected individuals. The amount of fluorescent dye indicating the number of CD3, CD4, or CD8 cells is expressed on the y-axis. The amount of dye
indicating the number of Fas antigen positive cells is expressed on the x-axis.

Figure 3 are graphs of a staining correlation of Fas- cells with disease progression as indicated by the absolute number of CD4+ cells. The percent Fas+ cells among CD5+, CD4+, and CD8+ cells is expressed on the y-axis. The total number of CD4+ cells (per mm³) is expressed on the x-axis.

Figure 4A-F present histograms showing the amount of Fas ligand expressed on the surface of monocytes for HIV infected patients (4B-F) and for a healthy volunteer (4A). Cells were analyzed by flow cytometry after staining for Fas ligand using an anit-Fas ligand monoclonal antibody (FL) or a control antibody (IgG1). The x-axis represents increasing fluorescence/intensity on a log scale. The y-axis represents the number of cells.

Figure 5 presents graphs showing the production of HIV in peripheral blood mononuclear cells (PBMC) after treatment with anti-Fas IgM (solid triangles) or control IgM (open triangles) as determined by a p24 assay. The x-axis represents days of incubation with PHA blasts. The y-axis represents p24 in pg/ml.

Figure 6 presents bar graphs showing the PHA stimulated proliferation of PBMC from two of the patients described in Example 5 after treatment with anti-Fas IgM or control IgM. The solid rectangles represent cells treated with control IgM. Open rectangles represent cells treated with anti-Fas IgM. The x-axis represents the
patient. The y-axis presents tritiated thymidine incorporation into cellular DNA in cpm.

Figure 7 is a graph depicting the Fas ligand activity of K562 cells transfected with human Fas ligand (K562 huFas ligand). The x-axis indicates the effector (K562 cells) to target (Jurkat cells) ratio. The y-axis shows the percent specific lysis. Solid squares represent K562 cells transfected with huFas ligand. Open squares represent control K562 cells. Open circles represent K562 cells transfected with huFas ligand and exposed to control supernatant. Solid diamonds represent K562 cells transfected with huFas ligand and exposed to HSV-1. Solid triangles represent K562 cells transfected with huFas ligand and exposed to HSV-2.

Description of the Preferred Embodiments

The present invention presents methods for the treatment and monitoring of diseases associated with a deficiency of Fas ligand activity. Such methods are based on establishing levels of Fas ligand activity increased above levels observed in the diseased state. In a preferred embodiment of the claimed invention the disease is HIV disease and treatment is by increasing levels of apoptosis of T-cells utilizing Fas ligand or agonists of the Fas antigen. This directly contradicts the prevailing view of those skilled in the art, who propose that increased cell death of T-cells accelerates the progression of HIV disease (Ameison, supra; Ameison and Capren, supra).
All techniques utilized to produce the reagents and to carry out the methods of the current invention are well known to those who practice the art.

**Example 1: Defective Fas ligand activity in PBMC of HIV infected individuals.**

Peripheral blood mononuclear cells (PBMC) from patients infected with HIV and from healthy volunteers were isolated and tested for Fas ligand activity. PBMC from a healthy control and HIV infected patients were isolated by ficoll/hypaque gradient centrifugation and washed in phosphate buffered saline with 0.5% bovine serum albumin and 0.01% sodium azide. Cytotoxicity Jam assays (Sieg, S. et al., *J. Virol.*, **62**:3538-3541, 1995; Matzinger, P. J. *Immunol. Meth.*, **145**:185-192, 1991) were performed with Jurkat cells (10⁶ ml⁻¹ in RPMI 1640 with 10% fetal bovine serum) labeled with tritiated thymidine (2 μCi ml⁻¹) for 4 hours at 37°C. Two μg/ml of either an anti-Fas monoclonal antibody that inhibits apoptosis mediated through the Fas ligand/Fas pathway (Immunotech) (this antibody is distinct from the anti-Fas antibodies useful in the current invention as it binds the Fas antigen but does not result in a signal for apoptosis) or an isotype matched control monoclonal antibody (IgG1) were added to the Jurkat cells during the last hour of this incubation. The target cells were washed and added to PBMC along with 500 ng of antibody. After 14 hours incubation at 37°C the cells were harvested and radioactivity determined by liquid scintillation. Anti-Fas monoclonal antibody used to identify the
component of the killing dependent on the Fas ligand/Fas pathway was able to completely inhibit apoptosis of Jurkat cells mediated by a cell line transfected with human Fas ligand, thus indicating the specificity of the inhibition mediated by the inhibitory anti-Fas monoclonal antibody. Percent specific apoptotic death was calculated by subtracting the experimental cpm from the spontaneous cpm, dividing this number by the spontaneous cpm and multiplying by 100.

Representative results (Figure 1A) demonstrate that PMBC from patients infected with HIV were markedly depressed in their capacity to mediate Fas ligand activity compared to PBMC from healthy volunteers. In aggregate, PBMC from HIV+ patients were approximately 70% to 75% decreased in Fas ligand activity (Figure 1B).

Approximately one-half of the patients did not demonstrate any Fas ligand activity. It is important to note that several of the patients with a complete absence of Fas ligand activity possessed relatively high numbers of CD4+ T-cells, i.e., greater than 400 cells/mm³, suggesting that the deficiency in Fas ligand activity was not the consequence of the clinical deterioration of the patients. In addition, the deficiency in Fas ligand activity did not correlate with disease progression (data not shown), thus the deficiency in Fas ligand activity may be an early consequence of HIV disease.
Example 2: Expression of Fas antigen on T lymphocytes from a healthy control and HIV infected individuals.

To see whether the capacity to mediate Fas ligand activity in vitro correlated with an effect in vivo, the number of Fas⁺ cells in the peripheral blood of patients infected with HIV was compared to a healthy volunteer. PBMC from a healthy control and 2 HIV infected patients were isolated by ficoll/hypaque gradient centrifugation and washed in phosphate buffered saline with 0.5% bovine serum albumin and 0.01% sodium azide. The cells ($5 \times 10^5$ ml⁻¹) were double-stained with fluoresceinated monoclonal antibodies specific for Fas antigen (2.5 μg ml⁻¹; Immunotech) and phycoerythrin conjugated monoclonal antibodies specific for either CD3, CD4, or CD8 (2.5 μg ml⁻¹; Dako). The cells were incubated with the antibodies for 30 minutes at 4°C, followed by 3 washes in staining buffer, and analyzed on a FACScan flow cytometer (Becton-Dickinson). The results demonstrate a marked increase in the relative number of Fas⁺ cells in HIV patients compared to healthy volunteers (Figure 2). This excess was found for CD3⁺ cells (all T-cells) and for both CD4⁺ and CD8⁺ T lymphocytes subsets. The excess of Fas⁺ cells in patients infected with HIV could be accounted for by an in vivo deficiency in Fas ligand activity as Fas⁺ T-cells would not be removed by the Fas ligand/Fas interaction.
Example 3: Correlation of Fas' cells with disease progression.

A further indication of the possibility of a disruption in the Fas ligand/Fas pathway of apoptosis is the increasing relative excess of cells expressing Fas antigen with disease progression as indicated by the loss of CD4⁺ T cells (Figure 3). Fas' cells are relatively protected from Fas ligand dependent killing as the disease progresses. As the number of CD4⁺ T lymphocytes decrease in patients the proportion of Fas' cells increases indicating that the defect in Fas ligand/Fas activity is involved in the pathogenesis of the disease.

The percent Fas' cells among the CD3⁺, CD4⁺, and CD8⁺ cells is plotted against the total number of CD4⁺ T lymphocytes which serves as an indicator for disease progression. Cells were stained and counted as in Example 2. Linear regression and r values were obtained by analysis via Cricket Graph software. For CD3⁺ cells r=0.778, for CD4⁺ cells r=0.773, and for CD8⁺ cells r=0.758.

Because there is an increase in the relative numbers of Fas' cells with disease progression, it is unlikely that accentuated killing of Fas' cells accounts for CD4⁺ cell loss in HIV disease. Conversely, the results suggest that the deficiency in Fas ligand activity may play a role in the pathogenesis of the disease.
Example 4: Defect in the expression of Fas ligand on monocytes isolated from HIV infected patients.

Staining of freshly isolated PBMC with anti-Fas monoclonal antibody followed by flow cytometry showed that Fas ligand was not expressed on any cells within the characteristic lymphocyte gates, which include lymphocytes and natural killer cells; however, the cells falling within the monocyte gates consistently showed moderate levels of Fas ligand expression (data not shown). Additionally, monocytes cultured without activation at 37°C showed markedly enhanced levels of Fas ligand surface expression and this enhancement was not affected by the presence of inhibitors of protein synthesis (data not shown). Thus, monocytes are responsible for the Fas ligand activity observed in unstimulated PBMC.

The monocyte fraction of PBMC from HIV infected persons was analyzed to determine whether the deficient natural Fas ligand activity mediated by their PBMC was related to decreased levels of Fas ligand surface expression on their monocytes. Monocytes in freshly isolated PBMC from five HIV infected patients were totally lacking surface Fas ligand (data not shown).

In another experiment, peripheral blood mononuclear cells were isolated from a healthy volunteer and from five HIV infected patients as described in Example 1 and were plated and incubated at 37°C for 1 hour. Nonadherent cells were collected and adherent cells were scrapped off the plates. Both adherent and non-adherent cells were stained with biotinylated NOK-1 (Pharmingen), a murine IgG1 anti-
human Fas ligand monoclonal antibody or a biotinylated control murine IgG1. After washing, the cells were incubated with streptavidin-RED670 (Gibco) and then analyzed by flow cytometry on a FACScan (Becton-Dickinson). Side scatter/forward scatter gates characteristic of monocytes were used to specifically analyze monocytes. Cell surface staining and flow cytometry were performed by standard techniques known to those who practice the art. (Keren et al., Flow Cytometry and Clinical Diagnosis, ASCP Press, Chicago, IL, 1994). Results are shown in Fig. 4. After an hour incubation at 37°C the monocytes isolated from HIV infected patients (Figs. 4B-F) showed little enhancement in Fas ligand surface expression compared to monocytes isolated from a healthy volunteer (Fig. 4A). Consequently, the defect in Fas ligand activity seen in PMBC from HIV infected persons is likely explained by the defect in surface Fas ligand expression on the patients’ monocytes.

Example 5: **Decrease in HIV viral titers by reconstituting Fas ligand in vitro.**

Peripheral blood mononuclear cells (PBMC) from 4 HIV infected patients were isolated as previously described, plated at $1 \times 10^6$ cells/ml and cultured at 37°C in the presence of either anti-Fas IgM (Immunotech) (100 ng/ml) or control IgM isotype control (100 ng/ml) for two days. The anti-Fas IgM is an agonist for Fas induced apoptosis, so that treatment with this antibody represents a reconstitution of Fas ligand activity in the patients’
PBMC. Following the incubation period, cells were recovered, washed and replated at $10^4$-$10^6$ cells/ml. PHA blasts derived three days prior from a healthy volunteer were added to the HIV infected PBMC at $0.5 \times 10^6$ cells/ml. At the times indicated in Figure 5, 1 ml of supernatant was removed, frozen at $-70^\circ$C and replaced with fresh media supplemented with 20 U/ml rIL-2 (Chiron). The supernatants were analyzed for HIV p24 content with an HIV-1 p24 antigen assay kit (Coulter Immunotech). The results are shown in Fig. 5. In the samples treated with anti-Fas IgM, HIV production was markedly inhibited.

To ascertain whether the treatment was detrimental to the function of the PBMC, cells treated with anti-Fas IgM or control IgM for two days were also tested for proliferative responsiveness to PHA. The cells were washed and replated at $10^5$ cells/well in 96-well flat-bottom plates. PHA was added at 2 μg/ml and cells were cultured at 37°C for 3 days. Wells were then pulsed with tritiated thymidine (0.5 μCi/well), and incorporation of tritiated thymidine into cellular DNA was assessed 18 h later (see Fig. 6). Treatment with anti-Fas IgM did not affect the capacity of the PBMC to proliferate. Thus, replacement of Fas ligand activity while significantly inhibiting HIV production does not reduce lymphocytic proliferation. Infected, poorly functional or nonfunctional cells are preferentially killed by anti-Fas, but functional cells are not damaged.

It has been shown that the inhibition of T cell apoptosis enhances the production of the virus and
facilitates persistent infection (Sandstrom et al., J. Virol. 70:4617, 1996; Antoni et al., J. Virol. 69:2384, 1995). While applicant is not bound by any one hypothesis, the data presented in this and other Examples lead to the hypothesis that HIV infection depresses Fas ligand activity in vivo and thereby enhances the survival of T cells that actively produce virus. It is possible that reconstitution of Fas agonism in vitro selectively induces the apoptosis of cells actively producing HIV, thereby accounting for the precipitous fall in viral production after a single treatment. The deficiency in Fas ligand activity may contribute to the pathogenesis of HIV disease by failing to eliminate a safe haven for the virus.

Example 6: Inhibition of Fas ligand activity by HSV-2.

Groups of K562 huFas ligand cells were exposed over-night to 3 plaque-forming units per cell of herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) or to an equivalent amount of control culture supernatant (cv-1). The cells were tested at a effector-to-target cell ratio of 5:1 at the highest concentration of effectors and serial 3-fold dilutions were performed. The target cells were tritium labeled Jurkat cells and a Jam cytotoxicity assay was performed as in Example 1. Results are presented in Figure 7. These data demonstrate a) that control K562 cells do not possess any capacity to kill the targets, b) that K562 huFas ligand efficiently kill the targets, c) that HSV-1 infection of K562 huFas ligand (or control
supernatant) does not affect this killing capacity, and d) that HSV-2 infection of the K562 huFas ligand cells markedly inhibited Fas ligand activity.

Example 7: Ex vivo treatment of PBMC

Leukocytes are removed by leukopheresis from a patient, such as an HIV infected individual. (Russell et al., Bone Marrow Transplant., 15:111, 1995; van Lunzen et al., Brit. J. Hematol., 88:46, 1994; Torpey et al., Clin. Immunol. Immunopathol., 68:263, 1993; Ho et al., Blood, 81:2093, 1993). The leukocytes are treated in a sterile fashion ex vivo with an appropriate concentration of an active Fas antigen agonist for a time sufficient to bind to the Fas-bearing cells (fifteen to thirty minutes) or time sufficient to bind and mediate apoptosis of the Fas-bearing cells (four to sixteen hours). Binding is carried out at 4°C in the presence of appropriate concentrations of sodium azide and exogenous protein such as human serum albumin or autologous human plasma or serum. Binding is determined by flow cytometric analysis with a labeled reagent that binds to the Fas agonist, such as fluoresceinated anti-Fas ligand antibody. Culturing for apoptosis is carried out at 37°C in the presence of appropriate culture medium such as RPMI 1640 with appropriate concentrations of exogenous proteins such as autologous human serum or plasma. Apoptosis can be determined by flow cytometric analysis with appropriate stain such as propidium iodide (Nicoletti et al., J. Immunol. Meth., 139:271, 1991). Fas antigen bearing cells
are assessed by flow cytometry with a labeled anti-Fas antibody that does not compete for binding to Fas antigen with the Fas agonist used. Examples of Fas antigen agonists include soluble Fas ligand (Tanaka et al., *supra*) or anti-Fas antigen antibodies. Fas antigen agonists not bound to the cells are washed away from the cells. Cell surface staining and flow cytometry are performed by standard techniques known to those who practice the art. (Keren et al., *Flow Cytometry and Clinical Diagnosis*, ASCP Press, Chicago, IL, 1994) The treated cells are returned to the patient's venous circulation by cellular infusion. (Riddell et al., *Science*, 257:238, 1992). *Ex vivo* treatment may be a preferred method based on the possible *in vivo* toxicity of Fas antigen agonists.

**Example 8: In vivo treatment with agonists for Fas antigen**

Agonists for Fas antigen are administered to patients, such as HIV infected patients, either intravenously, intramuscularly, subcutaneously, or intraperitoneally. The agonists include humanized anti-Fas antigen antibodies, soluble Fas ligand, an active extracellular portion of Fas ligand covalently attached to a targeting molecule, or cells that express Fas ligand activity. Doses are monitored for efficiency (the decrease in Fas antigen expressing T cells in the peripheral circulation), toxicity (adverse effects that influence the general well-being of the patient), and concentration of Fas antigen agonist in the blood. The decrease in Fas antigen expressing T cells is assessed by flow cytometry (see Example 2).
Toxicity is assessed by physical examination, blood chemistries (such as liver function tests), interview, and other well-established clinical modalities which are procedures well known to those skilled in the art. The concentration of Fas antigen agonist in the blood is determined by ELISA's (Jones et al., supra; Mariotti et al., supra) with specific antibodies for the Fas agonist. These procedures allow for an accurate assessment of the underlying defect and allow for optimization of the therapeutic dosing.

In order to minimize possible toxicity, Fas antigen agonists may be targeted to specific cells. HIV infected cells can be targeted by using antibodies specific for T cells, CD4+ T cells, or HIV infected cells. Targeting can be accomplished by combining agonist molecules that activate Fas antigen with molecules that bind to T cells, CD4+ T cells, or HIV infected cells. Molecules that bind to T cells include antibodies specific for CD3, CD5, CD7, CD8, CD2, or cytokines such as interleukin 2, interleukin 4, or interleukin 15. Molecules that bind to CD4+ T cells include antibodies specific for CD4. Molecules that bind to HIV infected cells include antibodies specific for gp120 or gp160 of HIV or soluble forms of CD4 including the extracellular portion responsible for binding to gp120 or gp160. Molecules that combine a Fas antigen agonist and a targeting molecules can be constructed by making bispecific antibodies, by crosslinking the different molecules, by covalently modifying the different molecules with complementary domains such as leucine zippers (Kos-
telny et al., J. Immunol. 148:1547, 1992) that can associate, or by attaching the molecules to the same substrate such as biodegradable bead or the same cell. Targeting molecules and Fas antigen agonists are attached to the same substrate by well-known chemical reactions utilized by those who practice the art. Attachment to the same cell can be carried out by methods of protein transfer or gene transfer. Targeting molecules are ligands for a cell surface molecule, other than Fas antigen, that is present on the target cell (i.e. the cell to undergo apoptosis).

Example 9: Determination of the Fas antigen activity and expression in patients

Peripheral blood is obtained form from patients such as those infected with HIV. Fas ligand activity is determined for isolated white blood cells or mononuclear cells using standard techniques such as cytotoxicity Jam assays using Jurkat cells labeled with tritiated thymidine as targets (see Example 1) or SupT13 or Fas antigen transfected cells as targets. Fas ligand activity by mononuclear cells is also determined by propidium iodide staining and flow cytometry using the same targets.

Fas ligand expression can be assessed by staining and quantitating Fas ligand expressing cells, such as monocytes. For example, this can be done by flow cytometry using specific anti-Fas ligand antibodies or a chimeric protein Fas-Fc or Fas-Ig (Alderson et al., J. of Exp. Med., 181:71, 1995) which consists of the extracellular component of Fas antigen combined with the Fc portion
of murine Ig. For example, leukocytes or mononuclear cells are isolated and treated with anti-Fas ligand antibodies or chimeric protein for fifteen to sixty minutes at 4°C in the presence of appropriate concentrations of sodium azide and exogenous protein such as bovine serum albumin. Unbound antibody or chimeric protein is washed away. The cells are stained with a fluorescently active molecule bound to an anti-mouse antibody. Flow cytometric analysis reveals the number of cells expressing Fas ligand. The anti-Fas ligand antibodies and the chimeric protein have specificity for human Fas. Alternatively, the anti-Fas ligand antibodies or chimeric proteins are directly labeled with a fluorescent molecule such as fluorescein and flow cytometric analysis proceeds after the initial incubation with the anti-Fas ligand antibodies. Another method comprises the use of biotinylated anti-Fas ligand antibodies followed by incubation with streptavidin conjugated to a reagent, such as RED670 (Gibco).

Monitoring is also accomplished by staining human peripheral blood mononuclear cells or leukocytes with anti-Fas antibodies using an analogous procedure as that described above for anti-Fas ligand staining.

**Example 10:** Genetic transfer and protein transfer of Fas ligand to patients with Fas ligand activity deficiency

Peripheral blood is obtained by venipuncture from, for instance, an HIV infected person. Mononuclear cells are
isolated from the blood using gradient centrifugation technology. The cells are treated with a form of Fas ligand or a portion thereof or agonistic anti-Fas antibodies or other Fas antigen agonists that allows for the molecules to adsorb to the cell surface in an active form. Activity is assessed with the methods described above. Adsorption is assessed with appropriate antibodies and flow cytometric analysis. Adsorption is enhanced by lipidating the molecules (Caras et al., *Science*, 328:1280, 1987; Tykocinski et al., *Proc. Natl. Acad. Sci. USA*, 15:3555, 1988) so that the lipid portions intercalate into the cells lipid bilayer, thereby causing the molecule to adhere to the cell. (Medof et al., *Biochemistry*, 25:6740, 1986; Huang et al., *Mol. Immunol.*, 31:1017, 1994) Alternatively, the cells are treated with Fas antigen agonists at high concentrations favoring nonspecific adsorption and cross-linking reagents are used to covalently attach the Fas agonists to the cells. Cells expressing Fas antigen agonists and Fas ligand activity are infused intravenously back into the patient in an appropriate physiological medium.

Alternatively, mononuclear cells from a patient are stimulated in culture with appropriate mitogenic agents such as anti-CD3 or phytohemagglutinin and grown in the presence of interleukin 2 for several months. During this time the cells expand in number and gene transfer is accomplished with any of a number of recombinant gene transfer vectors including selectable markers such as retroviral vectors. The vectors encode for the production
and expression of human Fas ligand (Tanaka et al., supra). After selection of the growing cells Fas ligand expression is assessed by flow cytometry and activity is assessed by methods described above. Cells expressing Fas ligand and Fas ligand activity are infused intravenously back into the patient in an appropriate physiological medium and in appropriate numbers.

Example 11: Associating domains

The leucine zipper portion of Fos and Jun provided in distinct chimeric molecules can be utilized to associate these molecules (Kostelny et al., supra). An expression vector encoding a Fas antigen agonist (for instance, soluble Fas ligand or heavy chain VH and CH1 regions of antibody with specificity for human Fas antigen) is made as a chimeric protein with the addition of the fos leucine zipper sequence. A heavy chain antibody with specificity for CD3 is constructed via an expression vector and the jun leucine zipper sequence is appended in place of the CH2 and CH3 domains to make a unique chimeric protein. For the chimeric heavy chain molecules, the expression vectors are co-transfected with an expression vector for the light chains of the antibodies into an appropriate cell line such as Sp2/0, a murine myeloma cell line. Antibodies are purified from culture supernatants using standard techniques. Fos and jun leucine zipper proteins preferentially associate with each other to form heterodimers. Signal transduction of the Fas antigen agonist chimeric leucine zipper molecule is regulated at
the cell surface by the presence of anti-CD3 antibody with an associating leucine zipper sequence. Alternatively, fos and jun leucine zipper sequences may be added to different aliquots of Fas antigen agonist. Use of these associating chimeric proteins allows for more regulation over signal transduction.

Modes of Administration

General methods and modes of administration are well known to those who practice the art and can be found in WO/95/25166, published September 21, 1995.

In treating a patient exhibiting a disorder of interest, a therapeutically effective amount of a agent or agents such as these is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of
circulating concentrations that include the ED$_{50}$ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC$_{50}$ as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by ELISA, activity assays, or flow cytometric assays.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1).

It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods.
Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

All references mentioned herein are incorporated by reference in their totality (including drawings).

Other embodiments are within the following claims.
CLAIMS:

1. Method for treating a patient with a condition characterized by a deficiency of Fas ligand activity comprising the step of administering to said patient an agent that increases the Fas ligand activity in said patient.

2. Method for treating a viral infection in a patient comprising the step of administering to said patient an agent that increases apoptosis of Fas' cells in said patient.

3. The method of claim 2 wherein said viral infection is caused by the human immunodeficiency virus.

4. The method of claim 2 wherein said viral infection is caused by herpes simplex virus type 2.

5. The method of claim 1 or 2 wherein said agent comprises a portion of the Fas ligand or another agonist of the Fas antigen sufficient to trigger apoptosis of said Fas' cells.

6. The method of claim 1 or 2 wherein said agent comprises an anti-Fas antibody.

7. The method of claim 6 wherein said antibody is humanized.
8. The method of claim 6 wherein said antibody is linked to a toxin.

9. The method of claim 2 wherein said agent comprises a bispecific antibody comprising a domain specific for Fas antigen and a domain specific for a cell surface marker located on the infected cell.

10. The method of claims 5 wherein said agent further comprises a targeting molecule.

11. The method of claim 1 or 2 wherein said agent comprises a Fas antigen agonist and a first associating domain and a targeting molecule and a second associating domain.

12. The method of claim 5 wherein said Fas ligand or Fas antigen agonist is provided by protein transfer to peripheral blood cells of said patient.

13. The method of claim 5 wherein said Fas ligand is provided by gene expression of Fas ligand in peripheral blood cells of said patient.

14. Method for determining when to initiate Fas ligand replacement therapy in a patient comprising the step of determining Fas ligand activity in said patient.
15. Method for determining when to initiate Fas ligand replacement therapy in a patient comprising the step of determining the presence or amount of Fas ligand in said patient.

16. Method for determining when to initiate Fas ligand replacement therapy in a patient comprising the step of determining the presence or amount of Fas antigen in said patient.

17. Method for monitoring Fas ligand replacement therapy in a patient comprising the step of determining Fas ligand activity in said patient.

18. Method for monitoring Fas ligand replacement therapy in a patient comprising the step of determining the presence or amount of Fas ligand in said patient.

19. Method for monitoring Fas ligand replacement therapy in a patient comprising the step of determining the presence or amount of Fas antigen in said patient.

20. The method of claim 1 wherein said agent comprises a bispecific antibody comprising a domain specific for Fas antigen and a domain specific for a cell surface marker located on the targeted cell.
FIG. 1A-1.
FIG. 1B.

- % FAS LIGAND DEPENDENT KILLING
- HEALTHY VOLUNTEERS
- HIV+ PATIENTS

- 25.8
- 6.5

EFFECOR PBMC
FIG. 6.

FIG. 7.

SUBSTITUTE SHEET (RULE 26)
# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

| IPC(6)     | A61K 39/00, 39/40, 39/42, 39/44, 39/395; G01N 35/567 |
| US CL.     | 424/198.1, 133.1, 183.1, 141.1, 143.1 |

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.       | 424/198.1, 133.1, 183.1, 141.1, 143.1 |

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)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>Y</td>
<td>JONES et al. Variations in Serum Soluble IL-2 Receptor Concentration. Pediatric Allergy and Immunology. November 1994, Vol. 5, No. 4 pages 230-234, see entire abstract.</td>
<td>15, 18</td>
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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 of particular relevance
  - "E" earlier document published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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  - "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
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**Date of the actual completion of the international search**

20 DECEMBER 1996

**Date of mailing of the international search report**

15 JAN 1997

**Name and mailing address of the ISA/US Commissioner of Patents and Trademarks**

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Form PCT/ISA/210 (second sheet)(July 1992)*
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<td>Y</td>
<td>CHAUDHARY et al. A Recombinant Immunotoxin Consisting of Two Antibody Variable Domains Fused to <em>Pseudomonas</em> Exotoxin. Nature. 01 June 1989, Vol. 339, pages 394-396, see whole article.</td>
<td>8</td>
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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, USPATFUL, WPIDS, MEDLINE, BIOSIS, EMBASE, CA, CAPLUS, CAPREVIEWS, BIOTECH
search terms: Fas antigen or ligand; apoptosis or cell death; viral infection or HIV or HSV or herpes simplex virus;
APO 1 or CD95;