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(54) Title: LEUKOTOXIN COMPOSITIONS AND THERAPEUTIC METHODS

(57) Abstract: A composition comprising leukotoxin proteins isolated from a bacterium is provided. In this composition, greater than 85% of the leukotoxin proteins are chemically modified at a basic amino acid residue, and the proteins induce cell death in myeloid leukocytes, while remaining substantially non-toxic to lymphoid leukocytes, lymphocytes, and red blood cells. Also provided is a method of selectively inducing cell death in myeloid leukocytes. The method comprises contacting the myeloid leukocytes with a composition comprising leukotoxin proteins. These leukotoxin proteins may be isolated from the NJ4500 strain of Actinobacillus actinomycetemcomitans. A method of purifying leukotoxin protein from the NJ4500 strain of Actinobacillus actinomycetemcomitans is also provided.

LEUKOTOXIN COMPOSITIONS AND THERAPEUTIC METHODS

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Provisional Application No. 60/739,537, filed November 25, 2005, the contents of which are incorporated herein by reference.

RELATED FEDERALLY SPONSORED RESEARCH

[002] The work described in this application was sponsored by the following The National Institute of Dental and Craniofacial Research, grant number R01 DE16133.

FIELD OF THE INVENTION

[003] The present invention is directed to a repeat in toxin (RTX) molecule that demonstrates leukocyte specificity and uses thereof. More particularly, the invention is directed to a leukotoxin that specifically targets myeloid leukocyte cells.

BACKGROUND OF THE INVENTION

[004] Bacteria and their toxins have been investigated for their anticancer activities. In the 1970s, bacteria (such as non-pathogenic Clostridium) were used for the treatment of malignant brain tumors, but the tumors recurred in these brain tumor patients. More than 100 microorganisms have been studied for their potential anticancer activities, and many bacteria have growth specificity for tumors that is 1000 times greater than for other tissue.

[005] While their anti-tumor activities make many bacteria attractive therapeutic agents, there are inherent risks to administering live bacteria to humans. A safer and more effective strategy has been to use biological toxins, specifically from bacteria, as therapeutic agents. Bacterial toxins are not only toxic, but are also highly specific for certain cell types, or can be engineered to be specific by fusing the toxin to other molecules. Many bacterial toxins are able to enter mammalian cells where they exert their toxic effects. Because of extensive evolutionary adaptation between bacteria and their hosts, bacteria have become very good at "developing" highly effective toxins.

[006] Each year, more than 60,500 people die of hematologic malignancies (leukemia, lymphoma, myeloma) with more than 110,000 new annual diagnoses in the US alone. Current treatment for these cancers includes the use of synthetic compounds that target the cell division process of nearly all cells of the body, not just the cancerous ones. As a result, devastating side effects are all too common. Furthermore, a significant percentage of patients eventually show resistance to many of the drugs, thus rendering treatment largely ineffective. Indeed, there is an effort to identify agents that induce cancer cell death by methods other than damage to DNA or cell division.

[007] While the drugs currently in use are toxic for cells, they are not highly specific. A new class of therapeutic agents for the treatment of hematologic malignancies, and cancer in general, includes drugs that exhibit specificity for predominantly the cancerous cell type. Examples of targeted therapeutics include Rituximab, which is a monoclonal antibody against B-lymphocytes, and Mylotarg, an antibody-anti-tumor antibiotic fusion directed against cells of myelomonocytic lineage.

[008] *Actinobacillus actinomycetemcomitans* is a Gram negative pathogen that inhabits the oral cavities of humans. *A. actinomycetemcomitans* is the etiologic agent of localized aggressive periodontitis (LAP), a rapidly progressing and destructive disease of the gingiva and periodontal ligaments. Among its many virulence factors, *A. actinomycetemcomitans* produces an RTX (repeats in toxin) leukotoxin. *A. actinomycetemcomitans* leukotoxin is an approximately 115 kDa protein that kills specifically leukocytes of humans and Old World Primates. Leukotoxin is part of the RTX family that includes E. coli α-hemolysin (HlyA) and Bordetella pertussis adenylate cyclase (CyaA). Leukotoxin may play an important role in *A. actinomycetemcomitans* pathogenesis by helping the bacterium destroy gingival crevice polymorphonuclear leukocytes (PMNs) and monocytes, resulting in the suppression of local immune defenses.

SUMMARY OF THE INVENTION

[009] A composition comprising leukotoxin proteins isolated from a bacterium is provided. In this composition, greater than 85% of the leukotoxin proteins are chemically modified at a basic amino acid residue, and the proteins induce cell death in myeloid leukocytes, while remaining

substantially non-toxic to lymphoid leukocytes, lymphocytes, and red blood cells. Also provided is a method of selectively inducing cell death in myeloid leukocytes. The method comprises contacting the myeloid leukocytes with a composition comprising leukotoxin proteins. These leukotoxin proteins may be isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans*. A method of purifying leukotoxin protein from the NJ4500 strain of *Actinobacillus actinomycetemcomitans* is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIGURE 1 shows fluorescence microscopy images of leukemia HL-60 cells when exposed to LtxA.

[0011] FIGURE 2 is a graph showing activity data of two forms of LtxA against human red blood cells.

[0012] FIGURE 3 is a series of images showing the effectiveness of NJ4500 LtxA *in vivo*.

[0013] FIGURE 4 a bar graph representation of data showing the sensitivity of human red blood cells to the JP2 and NJ4500 forms of LtxA .

[0014] FIGURE 5 is a bar graph representing data on the toxicity of the JP2 and NJ4500 forms of LtxA against HL-60 cells.

[0015] FIGURE 6 is a graph of time vs. cell death for various cell types in whole human blood during incubation with 0.2 μg/ml NJ4500 LtxA.

[0016] FIGURE 7 is a two-dimensional gel electrophoresis of two forms of LtxA.

DETAILED DESCRIPTION OF THE INVENTION

[0017] Leukotoxin is an effective cell-delivery protein, permeating leukemia cells and penetrating to the inside of specific cells. Leukotoxin mediated cell-delivery is demonstrated by introducing fluorescing molecules to specific cells, and measuring cell-delivery by monitoring the fluorescence by fluorescent microscopy. As shown in FIGURE 1, the

leukotoxin LtxA facilitates delivery of fluorescein into HL-60 leukemia cells. The leukotoxin forms pores or disruptions in the host cell membranes, and these openings in the membrane may allow the passage and entry of small molecules. In FIGURE 1, HL-60 cells were treated with fluorescein, a reagent that can be easily tracked by fluorescence microscopy. Fluorescein exhibits a green fluorescence color under the microscope, and is approximately the same molecular weight as many of the cancer drugs currently in use. The cells treated with leukotoxin (LtxA) and fluorescein (FIGURE 1, bottom panel) exhibited more intense and abundant fluorescence than the cells treated with fluorescein alone (FIGURE 1, center panel), indicating that leukotoxin is able to increase the number of fluorescein molecules that enter the cells.

[0018] Not only is leukotoxin capable of penetrating cells, but this penetration is toxic and lethal to HL-60 cells. HL-60 cells were modified to express luciferase genes, and with this HL-60*luc* system, it was shown that at certain concentrations, leukotoxin is quite toxic to the HL-60*luc* cells. By monitoring the luminescence of the cells, nearly 80% of the cells were killed by concentrations of leukotoxin as low as 200 ng/ml.

[0019] Data reflecting the sensitivity of HL-60*luc* cells to leukotoxin is shown in FIGURE 2. The activity of purified leukotoxin against HL-60*luc* cells in vitro is quantified. The leukotoxin used in this experiemenbt was LtxA isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans*. The LtxA was mixed with HL-60*luc* cells at various concentrations as indicated, and incubated for two hours, and then imaged with the IVIS 50 instrument. Relative viability was calculated by quantifying the number of photons produced in each well. Significant cell death was observed after two hours for concentrations of 2.0 μg/ml, 0.2 μg/ml, and 0.02 μg/ml.

[0020] To determine if the leukotoxin LtxA has activity *in vivo*, two Swiss Webster mice were injected i.p. with 10^6 HL-60*luc* cells. One of the mice was injected with 8 μg of LtxA i.p. immediately following HL-60*luc* cell injection. Both mice then received an i.p. injection of luciferin substrate. The mice were monitored by *in vivo* bioluminescence imaging with an IVIS 50 imaging system immediately following injection of the luciferin. The luminescent signal was visible and intense in the control mousee that did not receive the LtxA injection. In contrast, the mouse that received LtxA

showed essentially no luminescent signal, showing that the LtxA had killed the HL-60/*luc* cells in vivo.

[0021] Forms of LtxA include the JP2 form (isolated from the JP2 strain of *Actinobacillus actinomycetemcomitans*) and the NJ4500 form (isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans*). NJ4500 LtxA is well tolerated by the Swiss Webster mice. Two mice, weighing approximately 45 grams each, were injected with 10 μg of NJ4500 LtxA intravenously. These mice were monitored over a five-month period, and during this period, the mice remained healthy, did not lose weight, and had no apparent adverse reaction to the LtxA.

[0022] The forms of LtxA show different cell specificity. The NJ4500 and JP2 forms of LtxA demonstrate specificity to different types of blood cells, as demonstrated in FIGURE 4. The JP2 form of LtxA is lethal to human red blood cells, whereas human red blood cells are insensitive to the NJ4500 form of LtxA. Thus, the NJ4500 form of LtxA is lethal to HL-60 cells (as shown in FIGURE 3), but innocuous to human red blood cells (as shown in FIGURE 4). The JP2 form is quite lethal to human red blood cells, but as shown in FIGURE 5, is less deadly to leukemia cells than the NJ4500 form. The data displayed in FIGURE 4 was collected by a trypan blue dye excusion assay. Leukotoxin protein LtxA (2 μg/ml) was added to 1 X 10^6 HL-60 cells, and were incubated for 90 minutes at 37°C. The cells were measured for viability with the trypan blue dye exclusion assay.

[0023] The activity of the JP2 and NJ4500 forms of LtxA against HL-60 cells differs dramatically. In FIGURE 5, a bar graph representing the toxicity of the two forms of LtxA against HL-60 cells. As shown by the bar graph, the NJ4500 form of LtxA is much more lethal to the leukemia cell line than the JP2 form of the protein. Accordingly, not only is the NJ4500 form of LtxA non-lethal to human red blood cells (unlike the JP2 strain of LtxA), but the NJ4500 strain of LtxA is more lethal to leukemia cells than the JP2 strain of LtxA, thus indicating that the NJ4500 form of LtxA is a desirable leukemia or blood disease treatment as it is highly toxic to leukemia cells, but not to human red blood cells. LtxA provides a highly specific approach to treat hematologic malignancies, such as leukemia, lymphoma, and myeloma, without damaging other blood cells, such as red blood cells.

[0024] The data displayed in FIGURE 5 was collected using a trypan blue exclusion assay. HL-60 cells were mixed with 2 μg/ml final concentration

of LtxA from JP2 and NJ4500 as indicated and incubated for 90 minutes at 37 °C. Equal amounts of LtxA from either strain were mixed with approximately $5x10^6$ cells/ml of HL-60 cells and incubated for ninety minutes. Cell death was then assayed using the trypan blue dye exclusion assay. LtxA from NJ4500 was more effective at killing HL-60 cells than was LtxA from JP2. The toxin from NJ4500 was approximately twice as active and this result was highly reproducible for even different preparations of LtxA over four different experiments.

[0025] The NJ4500 strain of LtxA is also active in whole human blood. Whole human blood was mixed with LtxA (2.0 μg/ml final conc.) and incubated for 4 hours at 37 °C. The mixtures were then mixed with red blood cell (RBC) lysis buffer (eBioscience) and the RBCs were lysed according to the manufacturer's protocol. The remaining white blood cells (WBCs) were then resuspended in PBS and cells were counted using a ViCell counter (Beckman Coulter), which employs the trypan blue dye assay to measure viability. The sample that was not treated with LtxA had 93% viability, while the sample that was treated with LtxA had a viability of 42%. Because the RBC's were lysed and removed before viability was measured, the viability measurement assesses only the viability of the remaining white blood cells. The NJ4500 LtxA caused death in nearly 60% of the white blood cells.

[0026] NJ4500 LtxA displays unique sensitivity among the blood cells found in whole blood. At a concentration of 20 μg/ml, LtxA from NJ4500 showed a high level of specificity in inducing cell death in human whole blood. After four hours incubation at 20 μg/ml LtxA, the red blood cells, basophils, and lymphocytes suffered no significant cell death. In contrast, the 60% of the white blood cells were killed, and approximately 95% of the neutrophils, monocytes, and eosinophils were killed by the LtxA.

[0027] Similar specificity was shown over time at lower doses of NJ4500 LtxA. FIGURE 6 is a graph representing data that was collected over time, and shows that NJ4500 LtxA specifically target certain blood cell types. Neutrophil cells appear most sensitive to the relatively low concentration of 0.2 μg/ml LtxA with nearly 80% of neutrophils killed in only one hour. Both basophils and white blood cells (or leukocytes) were killed rapidly by the NJ4500 with about 60% of the cells dying within an hour. Monocytes are quite sensitive to NJ4500 LtxA, as nearly 100% of the monocytes died, however significant amounts of cell death required longer incubation

periods. In contrast, red blood cells (erthrocytes) and lymphcytes were completely insensitive to 0.2 μg/ml NJ4500 LtxA over time.

[0028] All histological data presented herein was collected through histology examinations. The blood samples were smeared onto a glass slide and then processed and stained on a Coulter LH slide maker, using a Wright stain for differentials.

[0029] The JP2 and NJ4500 forms of LtxA isolated *Actinobacillus actinomycetemcomitans* differ functionally in that the NJ4500 shows greater toxicity towards leukemia cells, along with greater specificity. The NJ4500 form of LtxA is highly specific towards white blood cells (leukocytes). Specifically, the NJ4500 form of LtxA is highly specific towards basophils, neutrophils, and monocytes. The NJ4500 form of LtxA is also highly specific towards eosinophils. This form does not induce significant cell death in red blood cells (erythrocytes) or lymphocytes. These functional distinctions may be related to structural modifications. Specifically, NJ4500 LtxA is highly modified with fatty acids.

[0030] A two-dimensional gel electrophoresis of JP2 and NJ4500 LtxA is shown in FIGURE 7. The gel shown in FIGURE 7 shows that the JP2 form of LtxA contains a significant amount of protein with an isolelectric point of at least 9.0. To create the 2-D gel, LtxA (20 mg) from JP2 or NJ4500 was separated first by isoelectric point through a pH gradient of 7–10. LtxA separated by isoelectric focusing was then separated by mass using polyacrylamide gel electrophoresis. Protein was visualized with SYPRO ruby stain. The LtxA samples were prepared for 2-D gel electrophoresis by processing 20 mg with a 2-D gel clean-up kit according to the manufacturer's directions (Amersham Biosciences, Piscataway, NJ). Following the clean-up, the pelleted protein was resuspended in 182 ml rehydration buffer (Bio-Rad, Hercules, CA) and mixed with 3.7 ml DTT (500 mg/ml) and 2 ml 1% bromophenol blue. The sample was then loaded onto a pH 7-10 IPG strip and processed for isoelectric focusing according to the manufacturer's protocol (Bio-Rad, Hercules, CA). After isoelectric focusing, the IPG strip was layered atop a 10% polyacrylamide gel and resolved in SDS buffer for several hours. Visualization of protein spots was accomplished by staining the gel in SYPRO ruby protein stain (Bio-Rad, Hercules, CA).

[0031] Two other RTX toxins, E. coli HlyA and B. pertussis CyaA, are modified covalently with fatty acid moieties at internal lysine residues. In

E. coli, hlyC is a fatty acyl transferase and is required for modification of HlyA. Based on the presence of an hlyC homologue in *A. actinomycetemcomitans* (ltxC), it is predicted that *A. actinomycetemcomitans* LtxA is also modified. Modification of HlyA and CyaA is required for toxin activity and the degree of modification is directly correlated with toxicity.

[0032] The two forms of LtxA were subjected to two-dimensional gel electrophoresis to assess whether differential modification of the proteins accounted for the functional differernces. The two-dimensional gel electrophoresis showed that LtxA from both strains exists in multiple isoforms (See FIGURE 7). Several representative spots were excised and subjected to trypsin digestion and MALDI-TOF MS analysis. MALDI-TOF MS confirmed that all species were *A. actinomycetemcomitans* LtxA (data not shown).

[0033] The predicted pI of LtxA based on primary amino acid sequence is approximately 9. (Represented by the rightmost spot and small arrow in Figure 7). Modification of lysine residues with fatty acids shifts the pI towards the acidic end, therefore, a greater fraction of the NJ4500 LtxA is modified compared to LtxA from JP2.

[0034] Approximately half of the JP2 LtxA is completely unmodified (as represented by the dense spot at approximately pI 9.). In contrast, none of the NJ4500 LtxA appears to be completely unmodified in the 2-D gel shown in FIGURE 7.

[0035] Other RTX toxins are modified with fatty acids and this modification is required for activity. Modification of RTX toxins may contribute to host and cell type specificity. E. coli, a-hemolysin, is modified at two internal lysine residues with C14, C15, or C17 fatty acid residues. Because E. coli can incorporate into HlyA three different fatty acids at two different lysine residues, preparations of HlyA are heterogeneous. Based on the two-dimensional gel electrophoresis data shown in FIGURE 7, LtxA is even more heterogeneous than HlyA.

[0036] LtxA has approximately 100 lysine residues and modification at several of them with different types of fatty acids accounts for the relatively large number of different isoforms. LtxA purified from NJ4500 is more active than LtxA from the JP2 strain. Nearly all of NJ4500 LtxA existed in some type of modified form whereas preparations of JP2 LtxA contained a significant amount of unmodified form (FIGURE 7). Consistent

with other RTX toxins, the unmodified toxin from JP2 is inactive against HL-60 cells. Thus, the percent of active LtxA molecules in preparations from NJ4500 is greater than from JP2.

[0037] A fatty acid is defined as a long-chain monobasic organic acid or a hydrocarbon chain. Fatty acids associated with NJ4500 LtxA were analyzed by Mylnefield Research Services, Lt., the Lipid Analysis Unit, Scotland. The protocol for analysis of myristoylated proteins described by Neubert, T.A. and Johnson, R.S. (Methods Enzymol., 250, 487-494 (1995)) was followed. It involves acidic hydrolysis of the protein, followed by acid catalysed methylation of the fatty acids for analysis by gas-chromatography. Approximately 3 mg of LtxA was subjected to this protocol, and preliminary data shows that at least myristic acid (C14) and palmitic acid (C16) are present in the LtxA protein.

[0038] The pI values of the two-dimensional gel indicated that basic residues are modified (making the protein more acidic). Any basic residue may be chemically modified by a fatty acid to reduce the pI. Because it is known that the lysine residues of RTX family members are modified by fatty acids, and that such a chemical modification increases activity of the RTX familiy members, the LtxA protein is likely to be modified at lysine residues.

[0039] Based on the data collected to date, the specificity and increased activity evident in the NJ4500 form of LtxA can be attributed to the composition of leukotoxin proteins with greater than 85% of the leukotoxin proteins chemically modified at a basic amino acid residue. This composition is an LtxA composition isolated from a bacterium such as *Actinobacillus actinomycetemcomitans*, and preferably the NJ4500 strain of *Actinobacillus actinomycetemcomitans*.

[0040] This composition as describes induces cell death in myeloid leukocytes. The composition is specific to any of white blood cells, neutrophils, monocytes, basophils, and eosinophils. Myeloid cells are cells belonging to the white blood cell lineage, and consist of granulocytes (basophils, neutrophils, eosinophils), monocytes, erythrocytes and platelets. The LtxA composition is specific as it is substantially non-toxic to lymphoid leukocytes.

[0041] White blood cells are a type of cell formed in the myelopoietic, lymphoid, and reticular portions of the reticuloendothelial system. Lymphocytes are a white blood cell formed in the lymphatic tissue

throughout the body (eg. Lymph nodes, spleen, thymus, tonsils, Peyer patches, and sometimes in the bone marrow). In normal adults approximately 22-28% of the total number of white blood cells in the circulating blood are lymphocytes.

[0042] The specificity of the NJ4500 LtxA is especially useful as a treatment against acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) because these are diseases in which only the myeloid cells are malignant. Thus, LtxA provides a high level of toxicity to certain myeloid cells, while leaving the red blood cell population unharmed, as the NJ4500 is substantially non-toxic to lymphocytes and red blood cells.

[0043] As discussed above, the NJ4500 LtxA composition of the invention includes chemical modifications, and the chemical modifications include fatty acid modifications to basic amino acid residues. Preferably, the basic amino acid residue that is modified is a lysine residue, and greater than 90% of the leukotoxin proteins are chemically modified at at least one basic amino acid residue.

[0044] Also discussed above, the NJ4500 LtxA compositions of the invention have a pI less than 9. Within the composition, 85% of the leukotoxin proteins have a pI less than 9.0. In another embodiment, 90% of the leukotoxin proteins have a pI less than 9.0, and in yet another embodiment, 95% of the leukotoxin proteins have a pI less than 9.0. In still another embodiment, 100% of the leukotoxin proteins have a pI less than 9.0.

[0045] As shown in FIGURE 7, most of the NJ4500 LtxA proteins have a pI less than 8.5. In one embodiment of the invention, 85% of the leukotoxin proteins have a pI less than 8.5, and in another embodiment, 90% of the leukotoxin proteins have a pI less than 8.5. The invention includes compositions where 95% of the leukotoxin proteins have a pI less than 8.5, and in yet another embodiment, 100% of the leukotoxin proteins have a pI less than 8.5. The leukotoxin proteins are isolated from *Actinobacillus actinomycetemcomitans,* and in another embodiments, the leukotoxin proteins are isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans.*

[0046] Because NJ4500 demonstrates a unique specificity among RTX family members, one embodiment of the present invention includes an RTX family protein that selectively lyses white blood cells more effectively

than red blood cells. The RTX family member is substantially non-toxic to red blood cells.

[0047] Also provided is a pharmaceutical composition comprising leukotoxin proteins and a pharmaceutically acceptable carrier, along with a method of selectively inducing cell death in myeloid leukocytes comprising contacting the myeloid leukocytes with a composition comprising leukotoxin proteins.

[0048] In another embodiment, a method of killing a target cell by contacting the target cell with leukotoxin proteins is provided. In this method each leukotoxin protein has a pI less than 9.0. In one embodiment of this method, the myeloid leukocyte cells die at a faster rate than lymphoid cells.

[0049] In still another embodiment, a method of treating a blood disease is provided. This method comprises administering a composition of leukotoxin proteins isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans* to a subject suffering from the blood disease. In treating a blood disease, a chemotherapeutic pharmaceutical may be administered to the subject in conjuction with the leukotoxin. Some appropriate chemotherapeutic pharmaceuticals include idarubicin, cytarabine, etosposide, daunorubicin, mitoxantrone, and melphalan. Other common chemotherapeutic agents for the treatment of leukemia and lymphoma include Chlorambucil, Fludarabine phosphate, Cytarabine, and Daunorubicin hydrochloride. These drugs share the common property of being highly toxic to humans, affecting many different tissue and organ systems of the body. Bone marrow suppression, severe neurologic effects, infertility, pulmonary, and gastrointestinal effects are some of the adverse effects exhibited by these drugs. Many of the drugs act by inhibiting DNA synthesis, a process that all dividing cells carry out. Most cells of the body are targeted by these non-specific pharmaceuticals. Any suitable pharmaceutical agent may be used in conjunction with LtxA, and the combination of a pharmaceutical agent with leukotoxin is intended to reduce the dose of the pharmaceutical necessary to achieve effective results in patients.

[0050] In addition to the potential uses as an anti-cancer agent, *Actinobacillus actinomycetemcomitans* leukotoxin may serve as a potent anti-viral. Specifically, HIV replicates and resides inside macrophages and T-lymphocytes. Viruses are difficult to combat because they often "hide"

from the immune system inside host cells. Leukotoxin could destroy those macrophages that are infected with HIV, allowing the virus to be released and attacked by the natural host immune defenses. This treatment would be different in that the therapy would not be directed against the virus (which would select for resistant HIV mutants), but rather against the host cell in which the virus resides.

[0051] In one embodiment the blood disease is leukemia, lymphoma, or myeloma, and in another embodiment, leukotoxin is used in a method of selectively sensitizing myeloid leukocyte cells to permeates. This method comprises contacting the myeloid leukocyte cells with a composition comprising leukotoxin proteins, wherein each leukotoxin protein has a pI less than 9.0, and lymphoid cells are substantially unsensitized to permeates by the composition.

[0052] In another embodiment, a method of purifying a RTX family protein from *Actinobacillus actinomycetemcomitans* comprises:

a. inoculating a single colony of *Actinobacillus actinomycetemcomitans* into a fresh broth and growing cultures;

b. adding the growing cultures to fresh broth, adding beads and incubating;

c. centrifuging the incubated culture, forming a pellet and a supernatant;

d. filtering the supernatant through a membrane to provided a filtered supernantant:

e. mixing $(NH_4)_2SO_4$ and the filtered supernatant together to form a mixture;

f. centrifuging the mixture to form a mixture pellet;

g. resuspending the mixture pellet in buffer to form a protein resuspension;

h. passing the protein resuspension through a column; and

i. collecting the protein eluting off the column.

EXAMPLES

Example 1: Purification of LtxA from the NJ4500 strain of *A. actinomycetemcomitans*

[0053] The JP2 strain of *A. actinomycetemcomitans* produces abundant LtxA, but it does not represent a fresh clinical isolate. Here, LtxA was purified from the clinical isolate NJ4500 of *A. actinomycetemcomitans*.

This strain also produces and secretes a large amount of LtxA, but the cells adhere to surfaces instead of growing planktonically. This type of adherent growth results in a relatively low number of cells per volume. The cell density of adherent cells was increased by increasing the surface area on which the cells can grow through the addition of spherical glass beads. Soda lime beads provided the greatest amount of LtxA when compared to Pyrex glass beads. The amount of LtxA that was purified from NJ4500 in the presence of soda lime beads was approximately twice that of JP2.

[0054] It is important to note that growth of *A. actinomycetemcomitans* in the presence of both types of glass beads was similar suggesting that differences in LtxA quantity was not due variable growth. *A. actinomycetemcomitans* strains JP2 and NJ4500 are known in the art. All bacteria were grown in *A. actinomycetemcomitans* growth medium (AAGM) as known in the art. Plates were incubated at 37 °C in 10% CO_2 for 4 days. Broth cultures were incubated for 24 h unless otherwise noted.

[0055] LtxA was isolated from JP2 by growing cells in 5 ml AAGM broth for 7–9 h and then diluted into 400 ml fresh AAGM broth. These cultures were then grown for 13–17 h before harvesting supernatant. To obtain supernatant, cultureswere centrifuged at 17,000 g for 10 minutes at 4 8C. The supernatant was filtered through a 0.22 mm low-protein binding membrane filter. For every 100 ml of filtered supernatant, 32.5 g $(NH_4)_2SO_4$ was added. The mixture was gently rocked at 4 8C for 1 h. The precipitated protein was collected by centrifugation at 10,000 g for 20 min at 4 8C. The pellet from 400 ml supernatant was then resuspended in 2 ml LtxA buffer (20 mM Tris-HCl, pH 6.8, 250 mM NaCl, and 0.2 mM $CaCl_2$).

[0056] The resuspended pellet was loaded on a column packed with 40 ml of Sephadex G-100 (Sigma, St. Louis, MO). Protein was eluted in 1 ml fractions with LtxA buffer. Protein content in each fraction was determined with the Bradford reagent. The three fractions with the highest protein content were combined, aliquoted and stored at -80 °C. The purity of LtxA was determined on a 4–20% SDS-PAGE gel and the concentration was determined by the BCA assay according to the manufacturer's protocol (Pierce, Rockford, IL).

[0057] LtxA was purified from the adherent strain NJ4500 by first growing cells in tubes filled with 5 ml AAGM broth for 14 h and then transferring 20 ml of growing cultures into 400 ml AAGM broth in a 500-ml bottle. Prior to adding 400 ml sterile AAGM broth to the 500 ml-bottle, 300 g of glass beads (or no beads, for controls) were autoclaved inside the bottle. The soda lime beads were obtained from Fisher Scientific (cat. 11-312C) and pyrex beads from Corning Incorporated (cat. 7268-5). The inoculated bottle was grown for 36–40 h as described above. During growth, the bottle was inverted several times to allow adherent cells to coat all the beads. After growth, the broth was removed and centrifuged and processed as described above for JP2 LtxA. For these experiments, cells were not removed from the beads.

Although adherent variants such as NJ4500 retain a greater amount of LtxA than the nonadherent variants, a large amount of secreted LtxA from NJ4500 can still be harvested. Because NJ4500 attaches avidly to surfaces, the number of growing cells per volume can be increased by adding 5 mm glass beads to the growth medium. In methods using one of two different types of glass beads, Pyrex and soda lime, the yield of LtxA from cells growing on Pyrex was significantly reduced when compared to the control of no glass beads or soda lime beads.

Example 2: Imaging of Mice Injected with HL-60luc

[0058] The images of the mice shown in FIGURE 3 were collected using *in vivo* bioluminescence imaging. The SCID mouse model has been used extensively for the study of hematologic malignancies, and the pattern of leukemia displayed in SCID mice closely resembles human clinical disease. In the model, leukemia cells are injected into SCID mice, usually intravenously. A commonly used leukemia cell line is HL-60, originally isolated from a 36-year-old female patient with acute promyelocytic leukemia. Animal studies have shown that HL-60 cells can infiltrate bone marrow, the spleen, thymus, kidney, liver, lungs, and even the brain. It has been reported that the mean survival time for SCID mice that were injected with HL-60 cells was 42.5 days following injection; however, this time can vary depending on the passage state of the HL-60 cells being injected.

[0059] *In vivo* bioluminescence imaging (BLI) is a technology that allows visualization of live bioluminescent cells (mammalian, bacterial, viruses) in a living animal, without sacrificing the animal. Cells to be visualized are engineered to express luciferase, which reacts with its substrate, to result in light production. Because the reaction also requires ATP, bioluminescence has also been used as a measure of viability. In bacteria, the substrate is encoded within the same operon as the luciferase enzyme. In the mammalian system, the substrate, luciferin, must be injected separately into the animal for the light-producing reaction to take place. Visualization of luminescent cells requires a highly-sensitive CCD camera that can detect low-level light within a short period of time. We currently maintain the Xenogen IVIS 50 imaging system for this purpose (Xenogen Corp., Alameda, CA). The distribution and abundance of luciferase-producing cells can be quantified by anesthetizing the animals and imaging them with the IVIS 50 imaging system.

[0060] BLI allows visualization of cells temporally and spatially, thus allowing small changes in cell number and localization to be detected over time. In contrast, using standard methods, animals must be sacrificed and extensive histological examination performed to localize cells in question. In general, the field of oncology has utilized BLI extensively, however, application of BLI to study hematologic malignancies has been limited, and to date, there are no bioluminescent hematologic cell lines commercially available (Xenogen Corp., Alameda, CA).

[0061] In the oncology models, engineered malignant cells are injected into animals and the progression of cancer is observed. In addition, transgenic light-producing mice are available for use in several oncology models (Xenogen Corp., Alameda, CA). Metastasis and regression can be monitored with great sensitivity and efficiency with the IVIS instrument. Of great significance, the effects of anticancer therapy can be determined before the endpoint of death is reached.

[0062] While preferred embodiments of the invention have been shown and described herein, it will be understood that such embodiments are provided by way of example only. Numerous variations, changes and substitutions will occur to those skilled in the art without departing from the spirit of the invention. Accordingly, it is intended that the appended claims cover all such variations as fall within the spirit and scope of the invention.

CLAIMS

What is Claimed:

1. A composition comprising leukotoxin proteins isolated from a bacterium, wherein greater than 85% of the leukotoxin proteins are chemically modified at a basic amino acid residue.
2. The composition of claim 1 wherein the bacterium is *Actinobacillus actinomycetemcomitans*.
3. The composition of claim 1 wherein the bacterium is a NJ4500 strain of *Actinobacillus actinomycetemcomitans*.
4. The composition of claim 1 wherein the proteins induce cell death in myeloid leukocytes.
5. The composition of claim 1 wherein the protein induces cell death in any one of white blood cells, neutrophils, monocytes, basophils, and eosinophils.
6. The composition of claim 1 that is substantially non-toxic to lymphoid leukocytes.
7. The composition of claim 6 that is substantially non-toxic to lymphocytes.
8. The composition of claim 1 that is substantially non-toxic to red blood cells.
9. The composition of claim 1 wherein the chemical modification is a fatty acid modification.
10. The composition of claim 1 wherein the basic amino acid residue is a lysine.
11. The composition of claim 1 wherein greater than 90% of the leukotoxin proteins are chemically modified at a basic amino acid residue.
12. A composition comprising leukotoxin proteins, wherein 85% of the leukotoxin proteins have a pI less than 9.0.
13. The composition of claim 12, wherein 90% of the leukotoxin proteins have a pI less than 9.0.
14. The composition of claim 13, wherein 95% of the leukotoxin proteins have a pI less than 9.0.
15. The composition of claim 14, wherein 100% of the leukotoxin proteins have a pI less than 9.0
16. A composition comprising leukotoxin proteins, wherein 85% of the leukotoxin proteins have a pI less than 8.5.

17. The composition of claim 16, wherein 90% of the leukotoxin proteins have a pI less than 8.5.
18. The composition of claim 17, wherein 95% of the leukotoxin proteins have a pI less than 8.5.
19. The composition of claim 18, wherein 100% of the leukotoxin proteins have a pI less than 8.5.
20. The compositions of claims 12-19 wherein the leukotoxin proteins are isolated from *Actinobacillus actinomycetemcomitans*.
21. The composition of claim20 wherein the leukotoxin proteins are isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans.*
22. An RTX family protein that selectively lyses white blood cells more effectively than red blood cells.
23. The RTX family protein of claim 22 that is substantially non-toxic to red blood cells.
24. A pharmaceutical composition comprising the composition of claim 1 and a pharmaceutically acceptable carrier.
25. A method of selectively inducing cell death in myeloid leukocytes comprising contacting the myeloid leukocytes with a composition comprising leukotoxin proteins.
26. The method of claim 25 wherein the leukotoxin proteins are isolated from *Actinobacillus actinomycetemcomitans*.
27. The method of claim 26 wherein the leukotoxin proteins are isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans.*
28. The method of claim 27 wherein the leukotoxin proteins are non-toxic to red blood cells.
29. A method of killing a target cell comprising contacting the target cell with a composition comprising leukotoxin proteins, wherein each leukotoxin protein has a pI less than 9.0.
30. The method of claim 29 wherein the leukotoxin proteins are isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans.*
31. The method of claim 29 wherein the target cell is a myeloid leukocyte cell.
32. The method of claim 31 wherein the myeloid leukocyte cells die at a faster rate than lymphoid cells.

33. A method of treating a blood disease comprising:
administering a composition of leukotoxin proteins isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans* to a subject suffering from the blood disease.

34. The method of treating a blood disease of claim 33 further comprising administering a chemotherapeutic pharmaceutical to the subject.

35. The method of treating a blood disease of claim 33 wherein the blood disease is leukemia or lymphoma.

36. A method of selectively sensitizing myeloid cells to permeates comprising contacting the myeloid cells with a composition comprising leukotoxin proteins, wherein each leukotoxin protein has a pI less than 9.0, and lymphoid cells are substantially unsensitized to permeates by the composition.

37. The method of claim 36 wherein each leukotoxin protein has a pI less than 8.5.

38. The method of claim 36 wherein the leukotoxin proteins are isolated from the NJ4500 strain of *Actinobacillus actinomycetemcomitans.*

39. A method of purifying a RTX family protein from *Actinobacillus actinomycetemcomitans* comprising:
a. inoculating a single colony of *Actinobacillus actinomycetemcomitans* into a fresh broth and growing cultures;
b. adding the growing cultures to fresh broth, adding glass beads and incubating;
c. centrifuging the incubated culture, forming a pellet and a supernatant;
d. filtering the supernatant through a membrane to provided a filtered supernantant:
e. mixing $(NH_4)_2SO_4$ and the filtered supernatant together to form a mixture;
f. centrifuging the mixture to form a mixture pellet;
g. resuspending the mixture pellet in buffer to form a protein resuspension;
h. passing the protein resuspension through a column; and
i. collecting the protein eluting off the column.

40. The method of purification of claim 39 wherein the protein is a leukotoxin protein.

41. The method of purification of claim 40 wherein the protein is a LtxA protein.

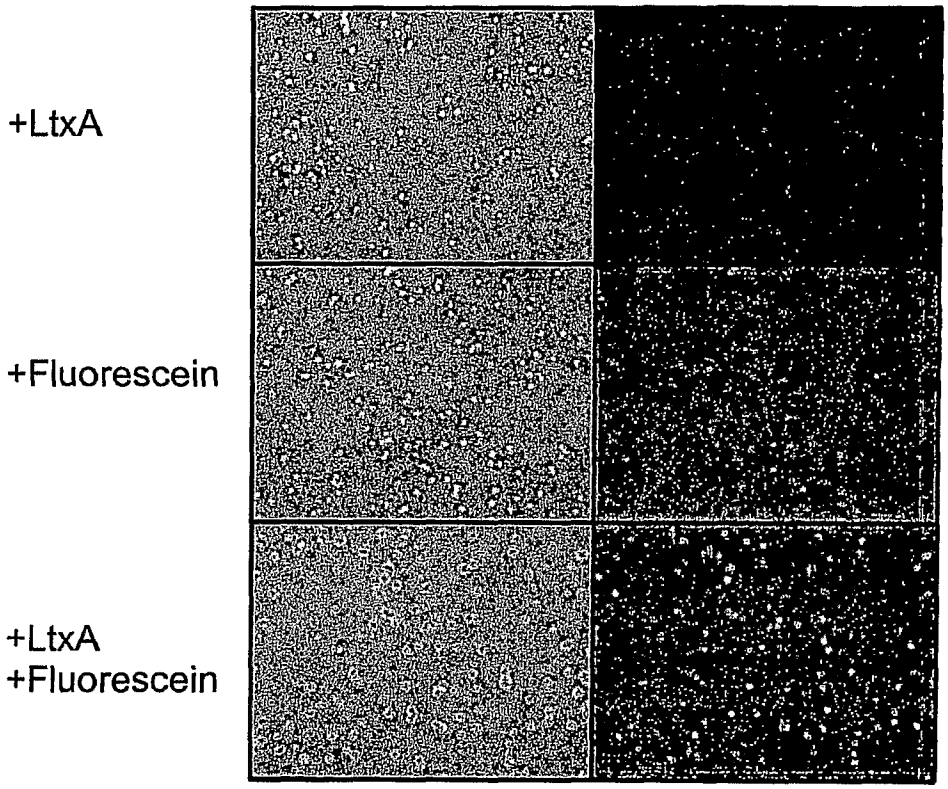

FIG. 1

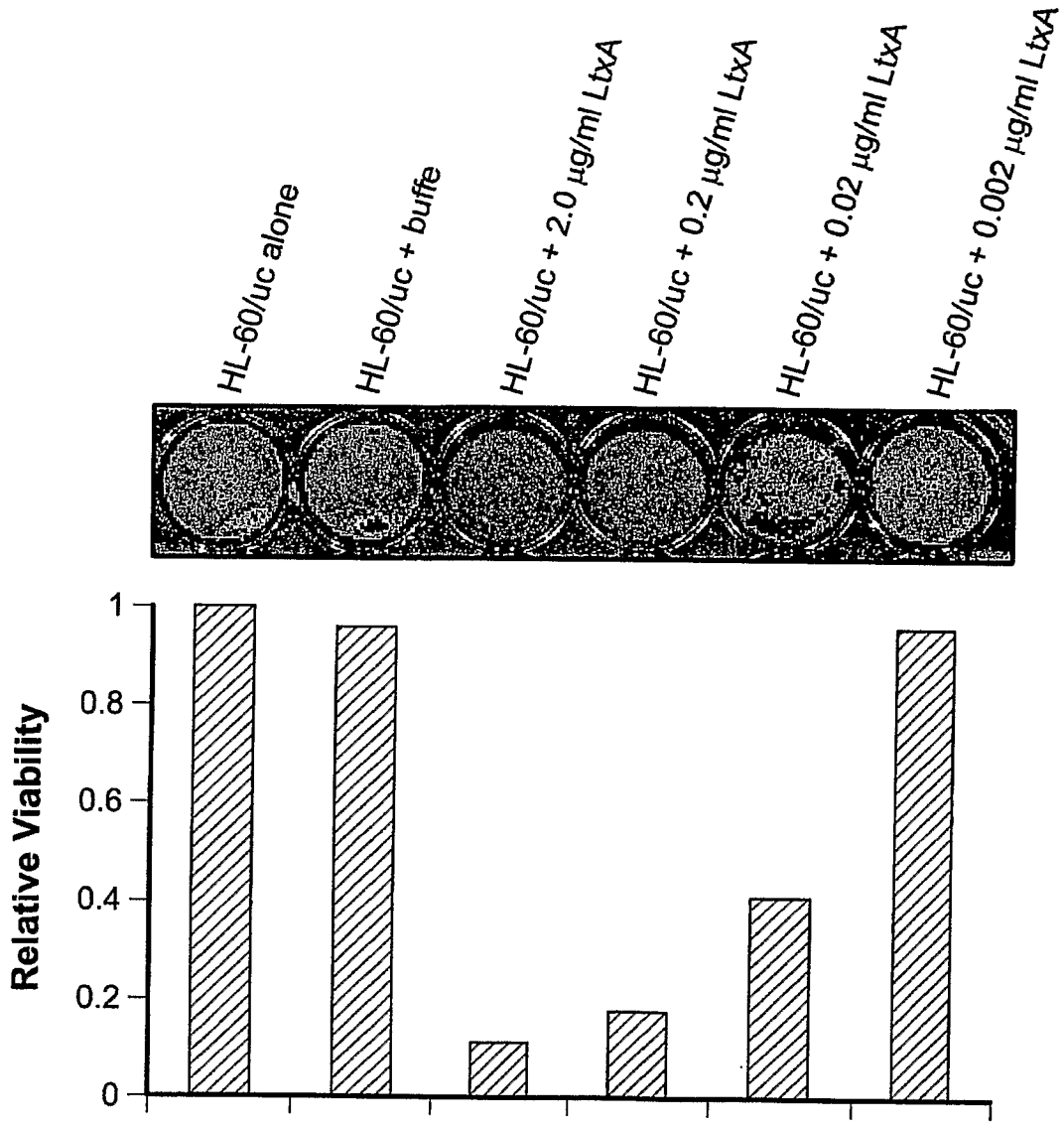
HL-60/uc alone
HL-60/uc + buffe
HL-60/uc + 2.0 µg/ml LtxA
HL-60/uc + 0.2 µg/ml LtxA
HL-60/uc + 0.02 µg/ml LtxA
HL-60/uc + 0.002 µg/ml LtxA
Relative Viability
1
0.8
0.6
0.4
0.2
0

FIG. 2

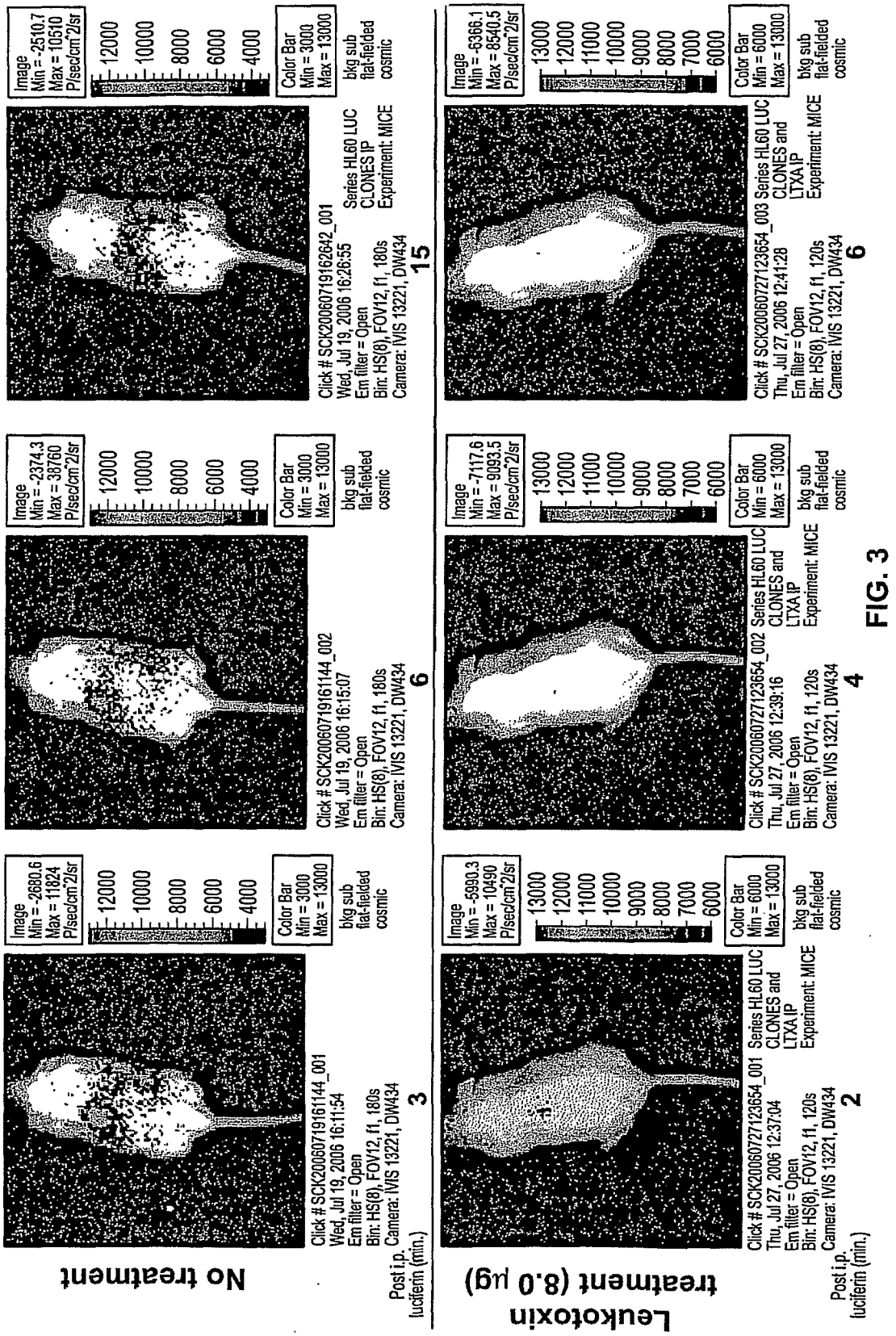
No treatment
Leukotoxin treatment (8.0 µg)
Image Min = -2680.6 Max = 11824 P/sec/cm^2/sr
Color Bar Min = 3000 Max = 13000
bkg sub flat-fielded cosmic
Click # SCK20060719161144_001 Wed, Jul 19, 2006 16:11:54 Em filter = Open Bin: HS(8), FOV12, f1, 180s Camera: IVIS 13221, DW434 Post i.p. luciferin (min.)
3
Image Min = -2374.3 Max = 38760 P/sec/cm^2/sr
Color Bar Min = 3000 Max = 13000
bkg sub flat-fielded cosmic
Click # SCK20060719161144_002 Wed, Jul 19, 2006 16:15:07 Em filter = Open Bin: HS(8), FOV12, f1, 180s Camera: IVIS 13221, DW434
6
Image Min = -2510.7 Max = 10510 P/sec/cm^2/sr
Color Bar Min = 3000 Max = 13000
bkg sub flat-fielded cosmic
Click # SCK20060719162642_001 Wed, Jul 19, 2006 16:26:55 Em filter = Open Bin: HS(8), FOV12, f1, 180s Camera: IVIS 13221, DW434 Series HL60 LUC CLONES IP Experiment: MICE
15
Image Min = -5990.3 Max = 10490 P/sec/cm^2/sr
Color Bar Min = 6000 Max = 13000
bkg sub flat-fielded cosmic
Click # SCK20060727123654_001 Thu, Jul 27, 2006 12:37:04 Em filter = Open Bin: HS(8), FOV12, f1, 120s Camera: IVIS 13221, DW434 Post i.p. luciferin (min.) Series HL60 LUC CLONES and LTXA IP Experiment: MICE
2
Image Min = -7117.6 Max = 9093.5 P/sec/cm^2/sr
Color Bar Min = 6000 Max = 13000
bkg sub flat-fielded cosmic
Click # SCK20060727123654_002 Thu, Jul 27, 2006 12:39:16 Em filter = Open Bin: HS(8), FOV12, f1, 120s Camera: IVIS 13221, DW434 Series HL60 LUC CLONES and LTXA IP Experiment: MICE
4
Image Min = -6366.1 Max = 8540.5 P/sec/cm^2/sr
Color Bar Min = 6000 Max = 13000
bkg sub flat-fielded cosmic
Click # SCK20060727123654_003 Thu, Jul 27, 2006 12:41:28 Em filter = Open Bin: HS(8), FOV12, f1, 120s Camera: IVIS 13221, DW434 Series HL60 LUC CLONES and LTXA IP Experiment: MICE
6

FIG. 3

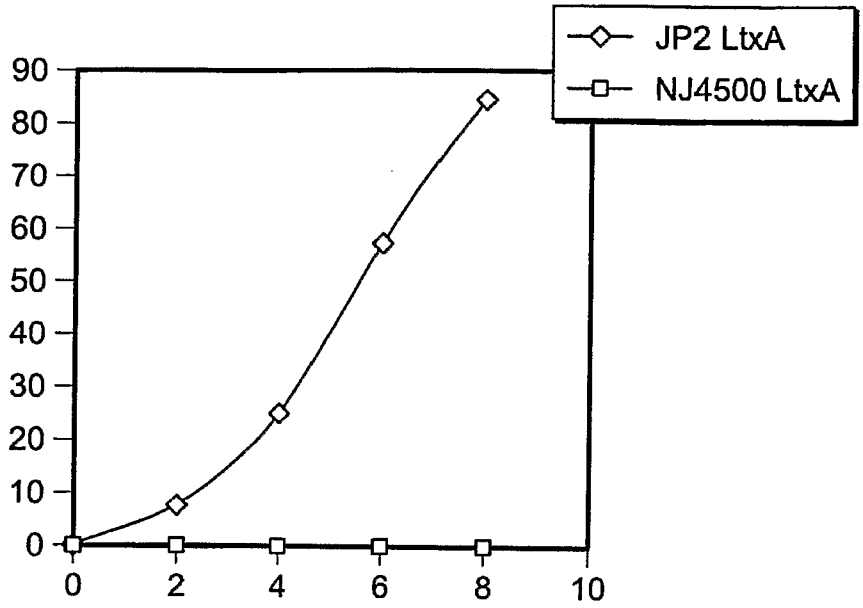
JP2 LtxA
NJ4500 LtxA
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10

FIG. 4

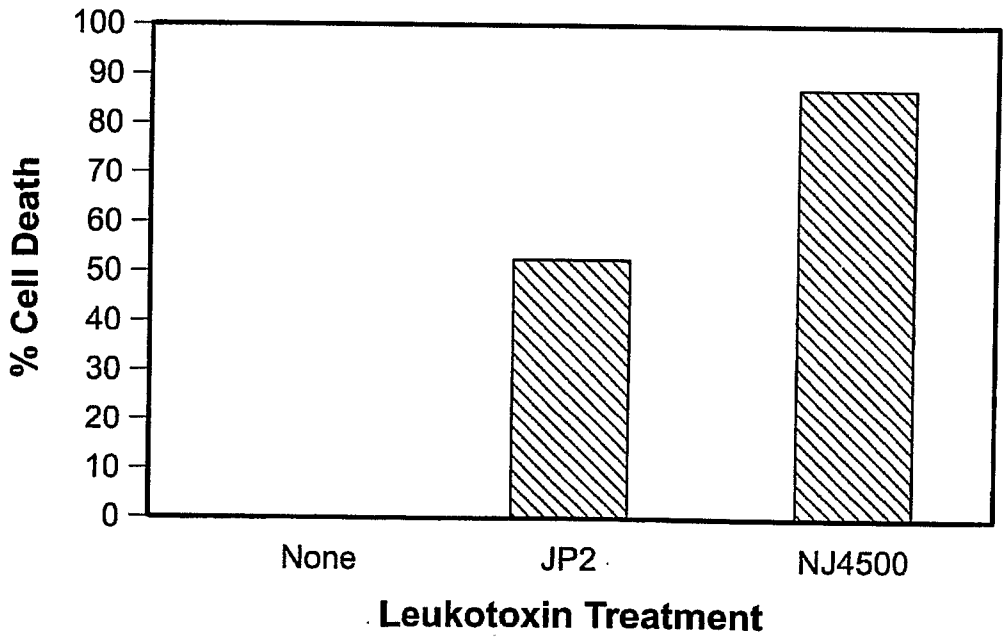
% Cell Death
100
90
80
70
60
50
40
30
20
10
0
None
JP2
NJ4500
Leukotoxin Treatment

FIG. 5

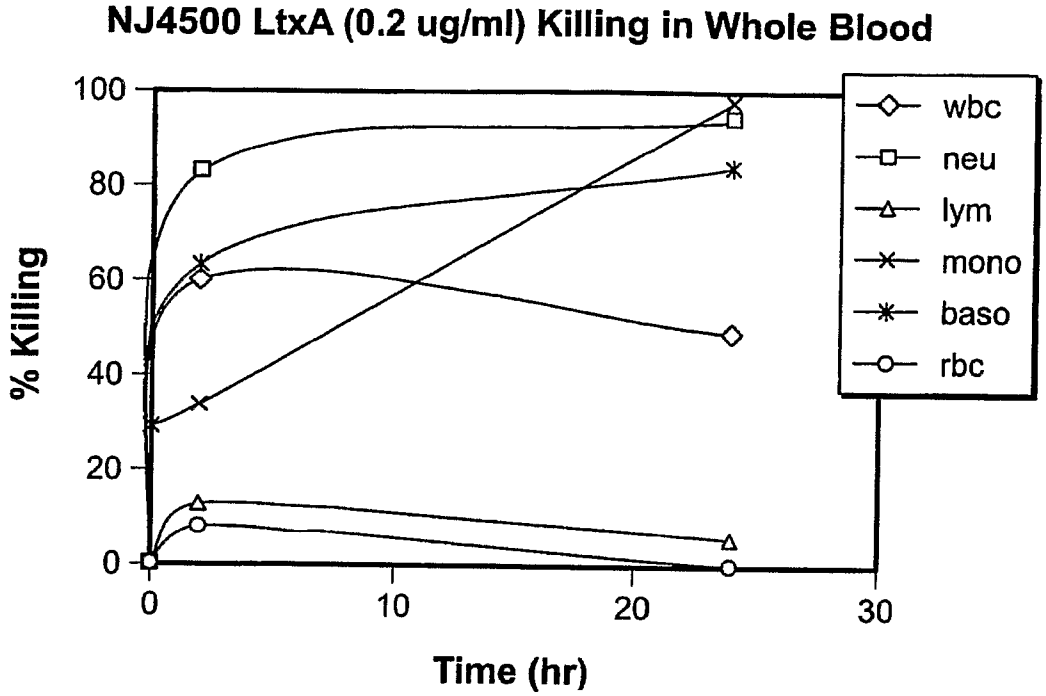
NJ4500 LtxA (0.2 ug/ml) Killing in Whole Blood
% Killing
100
80
60
40
20
0
0
10
20
30
Time (hr)
wbc
neu
lym
mono
baso
rbc

FIG. 6

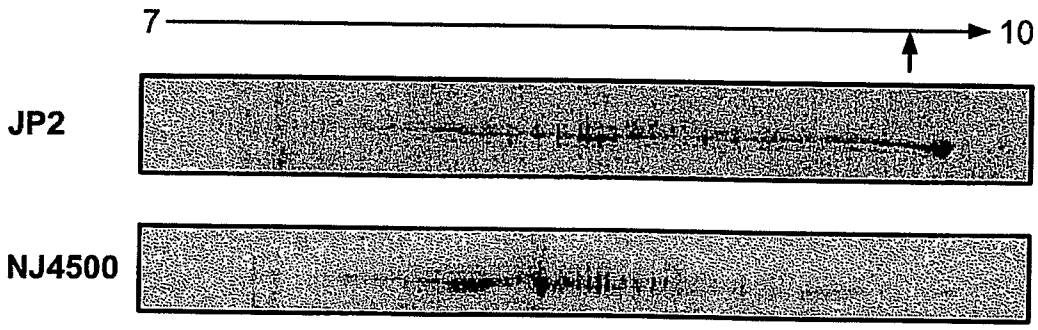
7
10
JP2
NJ4500

FIG. 7