Investigating the Lymphotoxic Activity of an Ancient Fungal Pathogen of Amphibians

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BRIEF: This research looked at how *Batrachochytrium dendrobatidis*, a fungal pathogen that infects amphibians, is able to suppress immune function.

ABSTRACT. Recently, global amphibian populations have dramatically declined. While many factors have contributed, one important cause is the chytrid fungus *Batrachochytrium dendrobatidis*. This evolutionary basal fungal pathogen produces factors which inhibit amphibian lymphocytes in culture. Inhibition is not limited to amphibian lymphocytes; proliferation of both murine and human lymphocytes has also been demonstrated. By inhibiting lymphocytes, *B. dendrobatidis* is able to evade host immune defenses. Previously, it has been shown that the fungus can cause apoptosis, which is the process of programmed cell death, in amphibian splenocytes. For this study, the ability of *B. dendrobatidis* factors to inhibit survival of the human T leukemia cell line Jurkat T cells was examined using an MTT proliferation assay. The mechanisms of inhibition were examined with flow cytometry which showed that Jurkat T cells were induced to undergo apoptosis when exposed to *B. dendrobatidis* supernatant in an apoptosis assay. To quantify apoptosis, two color flow cytometry was used to measure apoptosis after staining with fluorochromes, annexin V, and propidium iodide. Understanding the mechanism of death of leukemia cells induced by *B. dendrobatidis* factors may suggest possible new treatments for childhood cancers in addition to aiding understanding of this factor in global amphibian declines.

INTRODUCTION.

In the past two decades, there has been a marked decline in amphibian populations across the world [1]. While there are various causes for this decrease, ranging from climate change to habitat destruction, the fungus *Batrachochytrium dendrobatidis* is primarily responsible. [2]. It is a pathogenic and evolutionary basal fungus which has infected amphibian populations around the world. When it prevents the transportation of nutrients necessary for survival, such as electrolytes, the fungus causes chytridiomycosis, a fatal epidermal infection, which results in mortality rates of up to 100 percent [2].

Recently, it has been hypothesized that the lethality of *B. dendrobatidis* may be due to its ability to suppress the immune response from the animals it infects [3]. Immune responses from amphibians include an innate immune response and an adaptive immune response. Amphibians secrete amphibian antimicrobial peptides (AMPs) as a vital part of their non-specific innate immune response, which contributed to the first-line defense against *B. dendrobatidis* [4]. While there is much known about the innate immune response of amphibians, less is known about adaptive immune responses [4]. Adaptive immune responses are not able to adequately protect the infected animal from the fungus. Previous experiments have shown that water-soluble factors produced by *B. dendrobatidis* inhibited both amphibian and human lymphocytes and reduced viability of amphibian splenocytes [3]. One possible explanation for this is that proliferation was stopped via *B. dendrobatidis* factors inducing apoptosis in the lymphocytes and Jurkat T cells.

The aim of this research was to determine whether *B. dendrobatidis* factors induced apoptosis ultimately reducing the viability of Jurkat T cells. Apoptosis is a regular cellular process that is a form cell death. The process involves the degradation of the nucleus and cellular membrane as well as acidification of the cell. Understanding how *B. dendrobatidis* factors cause apoptosis in immune system cells is crucial in elucidating how the fungus is able to evade adaptive immune responses. Determining the link between proliferation, inhibition, and apoptosis of immune system cells as well as showing which event occurs first is crucial to the understanding of how the fungus is able to suppress the immune system of the organisms it infects.

When studying apoptosis in cell populations, it is important to identify characteristics of apoptosis in order to differentiate the process from necrosis, another type of cell death. Flow cytometry was used in order to achieve an accurate measurement of apoptosis in Jurkat T cells as previously shown [6,7]. The results show how *B. dendrobatidis* is able to suppress an amphibian's lymphocyte responses. Additionally, because this research was performed in a leukemia cell line, the results can also contribute to a possible treatment for cancer.

MATERIALS AND METHODS.

B. dendrobatidis supernatant preparation

To isolate water-soluble factors from *B. dendrobatidis*, cells were incubated at 21°C for 6 to 8 days in tryptone broth (T broth) (10 g tryptone/L). In order to prepare the supernatant which would contain the water-soluble factors, the cell culture was centrifuged at 1800-2000 RPM (~850 x g) for 20 minutes and the supernatant was then removed. Cells were then resuspended at 10^7 cells/ml in sterile glass distilled H_2O. After incubating for 24 hours, the culture was centrifuged at 1800-2000 RPM for 20 minutes. The supernatant was saved and filter sterilized. It was then frozen and lyophilized. Lyophilized powder was dissolved in RPMI medium in a volume corresponding to 1/10 the original volume of the *B. dendrobatidis* supernatant to generate a 10-fold increased concentration (10X) [3].

MTT Proliferation Assay

Jurkat T cells were passaged twice weekly and grown in RPMI medium (supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were spun at 1000 RPM (200 x g) for 10 minutes and were resuspended at $2x10^5$ cells/ml in RPMI medium. In order to test for proliferation, 50 µl of $2x10^5$ cells/ml in RPMI medium were added to 50 µl of either RPMI medium, *B. dendrobatidis* supernatant at 10X, or etoposide (25 µg/ml, a known inhibitor of Jurkat growth). Cells were cultured 3 days at 37° C with 5% CO_2-95% air. After incubation, cells were centrifuged for 20 minutes at 3000 RPM (~2200 x g) and the supernatant was removed. Then, 100 µl of MTT (thiazolyl blue tetrazolium bromide at 500 µg/ml) was added to each sample. The Jurkat T cells were incubated for another 2 to 4 hours at 37° C with 5% CO_2-95% air. This produced a purple crystal composed of MTT that formed in the sample. The samples were centrifuged for 30 minutes at 3000 RPM. The media of the samples were then removed, and 100 µl/well of DMSO was added to the samples. Samples were incubated until the cells dissociated. The optical density of the culture plates was then read at 570 nm with a spectrophotometer to measure absorbance.

Apoptosis Induction

Jurkat T cells were cultured in the presence RPMI medium for 6 to 8 days. The cells were then resuspended at $2x10^6$ cells/ml in RPMI. With each sample, 500 µl of medium, medium plus anti-Fas antibodies (positive control), or medium plus 5X *B. dendrobatidis* supernatant was added to 500 µl of Jurkat T cells at $2x10^6$ cells/ml in RPMI. The cells were then incubated at 37°C with 5% CO_2-95% air for 24 hours.

Propidium iodide (PI) Staining (to detect apoptosis)

After incubating for 24 hours, 1 x 10^6 cells were spun at 1000 RPM (~200 x g) for 10 minutes and washed with PBS. They were then fixed with 1 mL of ethanol fixative (9 parts 70% ethanol, 1 part PBS) and incubated at -20°C for 24-48 hours. Cells were spun and then resuspended in 0.5 ml propidium iodide (2 mg propidium iodide, 3.7 mg EDTA, and 0.1 ml TritonX-100 in 100 ml PBS) and incubated at room temperature in the dark for 30 minutes. After the incubation period, the cells were treated with RNase A (50 units/ml) for 15 minutes in the dark. [8]. Cells were then taken as quickly as possible to a flow cytometer for analysis [3].

PI and Annexin V Staining (to detect apoptosis)

To test for apoptosis, cells were cultured as previously described with medium plus anti-Fas antibodies or camptothecin (5 µM) (positive controls), or medium plus 5X *B. dendrobatidis* supernatant. This method was adopted from the BD Pharmingen FITC Annexin V Apoptosis Detection Kit. The plate was incubated at 37°C with 5% CO_2-95% air for 24 hours. Cells were then washed twice with PBS and resuspended with 1X Annexin V Binding Buffer [0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 5 mM $CaCl_2$] at a concentration of 1x10^6 cells/ml. From the binding buffer solution, 100 µl of the solution was removed and added to 5 µl of propidium iodide (PBS, pH 7.4) and 5 µl of FITC conjugated Annexin V (containing 0.09% sodium azide). The cells were vortexed and incubated at room temperature in the dark for 15 minutes. Afterwards, 400 µl of 1X Annexin V Binding Buffer was added to each sample. Apoptosis was measured in the samples as soon as possible using flow cytometry. Controls included an unstained sample, a propidium iodide only stained sample, and a FITC/Annexin V only stained sample to calibrate the flow cytometer (Fites, et al. 2013).

Flow cytometry

All cell samples were analyzed using flow cytometry to measure for apoptotic activity. Flow cytometry was able to determine apoptotic cells through the forward and side scatter results from the interaction between a cell and light source. Unstained controls were used to set up preliminary gates. These gates were used to quantify a specified portion of a cell population, such as apoptotic cells. Data was measured and recorded on either a five- or three-laser BD LSRII flow cytometer. Events that were measured from the flow cytometer were recorded and gated on BD FACSDiva Software.

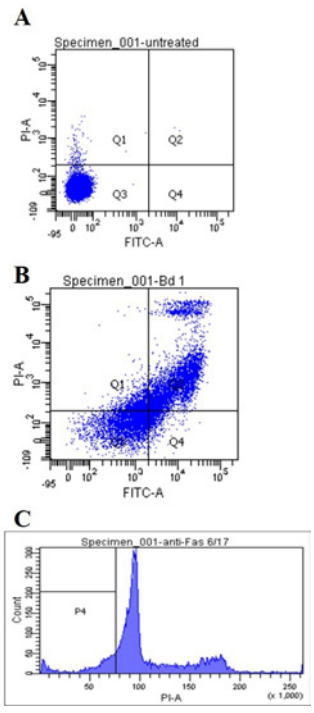

Figure 1. Flow cytometry plots. PI-A indicates the amount of cells stained by propidium iodide, FITC-A indicates the amount of cells stained by annexin V. (A). Untreated Jurkat T cells stained with PI and annexin V. Cells are mostly in quadrant 3, indicating that the population is not apoptotic (B) Jurkat T cells exposed to 5X *B. dendrobatidis* supernatant for 24 hours. Cells in quadrant 4 are in early apoptosis, and cells in quadrant 2 are in late apoptosis. (C) An example of what a flow cytometry plot would look like with PI staining. Percent apoptosis was determined by the P4 interval gate.

RESULTS.

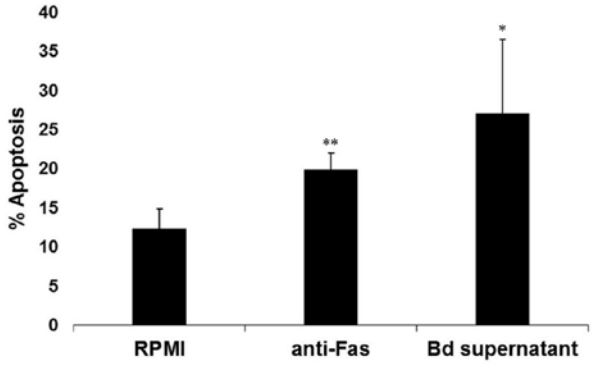

Figure 2. The average percentage of apoptosis in cell populations as shown by PI staining (N=7). Cells were incubated with either RPMI, anti-Fas, or 5X *B. dendrobatidis* supernatant for 24 hours. Cells were stained with propidium iodide, cells that showed less DNA stain than normal diploid cells were considered to be apoptotic. *B. dendrobatidis* and anti-Fas caused significant apoptosis when compared to cells in RPMI (*p<0.05, **p<.01).

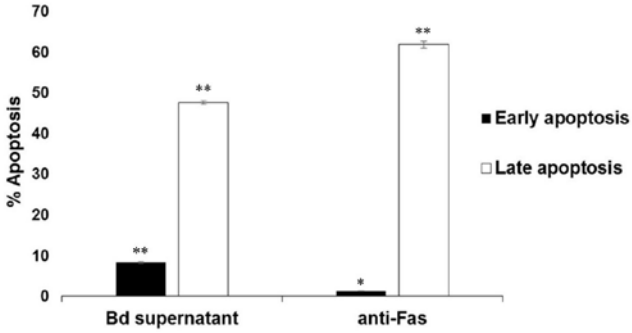

Figure 3. The average percentage of apoptosis in cell populations treated with *B. dendrobatidis* supernatant or anti-Fas antibody when stained with Annexin V and PI (N=20). Cells were incubated with either RPMI, anti-Fas antibodies, or 5X *B. dendrobatidis* for 24 hours. Cells that were stained with annexin V were considered to be in early apoptosis, and cells stained for both annexin V and propidium iodide were considered to be in late apoptosis. Both *B. dendrobatidis* and anti-Fas caused a significant proportion of cells to undergo apoptosis in Jurkat T cell populations when compared to the untreated control (*p<0.05, **p<.01).

DISCUSSION.

The focus here centered on learning how *Batrachochytrium dendrobatidis*, a fungal pathogen that has infected amphibian populations worldwide, is able to cause immune suppression. It was already known that the fungus is able to suppress immune system cells of the organisms it infects [3]. However, it was not known how this suppression occurred. The results here suggest that the fungus causes apoptosis in lymphocyte cell populations, and future experiments will determine whether this apoptosis occurs before or after proliferation is blocked.

The MTT assay shows that *B. dendrobatidis* was able to significantly decrease cell growth (Figure S1). The factors released by the fungus induced a dose-dependent inhibition with the concentrations tested, indicating that there is something produced by *B. dendrobatidis* that is able to kill these cells. The interesting aspect of this result was that *B. dendrobatidis* at 5X its original concentration was able to completely suppress Jurkat leukemia T cell growth similar to a chemotherapeutic drug, etoposide. This suggests that the factors produced by *B. dendrobatidis*, if isolated and further studied, could provide an alternative method for treating certain cancers due to its ability to inhibit cancerous immune cells by initiating apoptosis.

Both the PI-only and annexin V plus PI staining methods show that *B. dendrobatidis* was able to cause a significant amount of apoptosis in Jurkat T cells (Figures 1-3). There were differences between the two different staining techniques. The first used only propidium iodide, which was able to quantify cells with a reduced content of DNA. The problem with this method is that it also counts possible necrotic cells, a small percentage of the cells measured. In order to get a more accurate quantification of apoptosis in the Jurkat T cell populations measured, a two-dye staining technique with both PI and annexin V was used. Cells whose membranes were not degraded by apoptosis would be able to be stained with annexin V but not propidium iodide. Degradation of the cellular membrane is a characteristic of late apoptosis, meaning cells that were positive for annexin V and negative for propidium iodide were in the early stages of apoptosis. Annexin V is able to stain cells undergoing early apoptosis as it binds to phospholipid phosphatidylserine (PS), which is exposed and easily bound during the early stages of apoptosis. Both anti-Fas and *B. dendrobatidis* supernatant caused significant apoptosis when compared to cells incubated with RPMI media. Additionally, there was not a significant difference between anti-Fas and *B. dendrobatidis*. This was observed by both methods of the staining process.

Two different methods were used to quantify the amount of apoptosis in Jurkat T cell populations (Figure 2 and 3). Throughout multiple trials, the plots were reproducible. The plots were able to give a percentage of cells undergoing various stages, as seen in figures 2 and 3. This indicates that gates were set up properly, a key aspect of accurately measuring cell apoptosis from flow cytometry. Unstained controls remained consistent throughout the trials preformed as well.

Future studies should look at isolating factors of interest made by *B. dendrobatidis*. These toxins could be separated by liquid chromatography and analyzed to see what mechanisms are used to cause apoptosis in lymphocytes. Treatments could then be developed that target these toxins specifically [9]. Isolating these toxins could lead to the creation of a vaccine that could be administered that would help to protect amphibians against the suppressive effects of the fungus [10]. Additionally, future studies could look at different cells of the vertebrate immune system, such as B cells and other types of T cells to see if *B. dendrobatidis* has a similar effect on them. More studies looking at how different concentrations of *B. dendrobatidis* affect apoptosis in leukemia cells and whether they are more sensitive than normal lymphocytes would also be helpful in establishing a relationship between the amount of *B. dendrobatidis* factors produced in a natural setting and the amount of apoptosis that occurs.

In conclusion, the chytrid fungus *B. dendrobatidis* causes apoptosis in lymphocyte cells. This was shown by incubating Jurkat T cells with the fungus, and measuring the amount of apoptotic cells over a period of time. Additionally, it was shown that *B. dendrobatidis* is able to suppress the proliferation of Jurkat T cells in a dose-dependent manner. Further research looking at the toxic factors from *B. dendrobatidis* has the potential to yield a possible cancer treatment as *B. dendrobatidis* has the ability to inhibit immune system cells. There is a possibility that purified *B. dendrobatidis* factors could be used to target these cancerous immune system cells as well.

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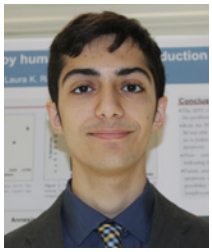
SUPPORTING INFORMATION.
Figure S1. Average OD reading from a MTT proliferation assay (N=5). Cells were incubated with either RPMI, etoposide (25 μg/ml), or decreasing concentrations of *B. dendrobatidis* supernatant for three days. Color was measured with a spectophotometer via adding DMSO to cells after incubation period. Positive control was RPMI media and the negative control was etoposide.

REFERENCES.
1. Wake DB and Vredenburg VT, PNAS. 105:11466-11473 (2008).
2. Voyles J, et al., Microbes Infect. 13:25–32 (2011).
3. Fites JS, et al., Science. 342:366-369 (2013).
4. Rollins-Smith LA, et al., Int Comp Biology. 51:552-562 (2011).
5. Nichols DK, et al., J Wildlife Diseases. 37:1–11 (2001).
6. Vermes I, et al., J Immunological Methods. 243:167-190 (2000).
7. Gottlieb RA, et al., PNAS. 93:654-658 (1996).
8. Riccardi C and Nicoletti I, Nature Protocols. 1:1458-1461 (2006).
9. Rosenblum EB, et al., Diseases of Aquatic Organisms, 92:131-147 (2009).
10. Stice EJ and Briggs CJ, J Immunological Methods. 46:70-77 (2010).



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