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Antiviral activity of serum from the American alligator (*Alligator mississippiensis*)

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Abstract

Serum from wild alligators was collected and tested for antibiotic activity against three enveloped viruses using cell-based assays. Alligator serum demonstrated antiviral activities against human immunodeficiency virus type 1 (HIV-1; IC_{50} = 0.9%), West Nile virus (WNV; IC_{50} = 4.3%), and Herpes simplex virus type 1 (HSV-1; IC_{50} = 3.4%). The inhibitory concentration (IC_{50}) is defined as the concentration of serum that inhibits 50% of viral activity. The antiviral effects of the alligator serum were difficult to evaluate at high concentrations due to the inherent toxicity to the mammalian cells used to assay viral activities. The TC_{50} (serum concentration that reduces cell viability to 50%) values for the serum in the HIV-1, WNV, and HSV-1 assays were 32.8, 36.3 and 39.1%, respectively. Heat-treated serum (56 °C, 30 min) displayed IC_{50} values of >50, 9.8 and 14.9% for HIV-1, WNV and HSV-1 viruses, respectively. In addition, the TC_{50} values using heat-treated serum were substantially elevated for all three assays, relative to untreated serum (47.3 to >50%). Alligator serum complement activity has been shown to be heat labile under these conditions. HIV-1 antiviral action was heat-sensitive, and thus possibly due to the action of serum complement, while the anti-WNV and anti-HSV-1 activities were not heat labile and thus probably not complement mediated.

Keywords: Alligator; Antiviral; Complement; Crocodilian; Serum

Alligators often sustain serious injuries due to territorial disputes with other alligators. These conflicts often end with severe damage such as loss of entire limbs. However, alligators exhibit remarkable ability to heal rapidly and without infection, particularly considering the septic environment in which they live (Merchant, unpublished observations). Recent studies in our laboratory have demonstrated the wide-ranging antibacterial and amoebacidal activities of alligator serum (Merchant et al., 2003, 2004). More recent studies have demonstrated that these activities are due to a potent and broad-acting serum complement system (Merchant et al., 2004). This study was conducted to investigate the antiviral effects of alligator serum.

Wild alligators were captured at night from a boat with the use of a spotlight and a cable snare. Blood was collected via the spinal vein using an 18 ga. needle and a 60 mL syringe (Olsen et al., 1975; Zippel et al., 2003). The blood was removed to a 15 mL serum Vacutainer® and allowed to clot at room temperature for approximately 3 h. The serum was separated by centrifugation (3000 × *g*, 15 min) and was frozen at −20 °C until ready for use.

The effectiveness of alligator serum against the laboratory-adapted IIIB strain of Human immunodeficiency virus-type 1 ($HIV\text{-}1_{IIIB}$) was examined using a cell-based assay in which the virus was used to infect a human T lymphoblastoid cell line (CEM-SS). The assays were conducted as previously described (Buckwold et al., 2004). The results represent the means of three independent determinations for each serum dilution and are expressed as % reduction of viral cytopathic effects (CPE), based on virus control (cells plus virus, no

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drug; 0% baseline) and cell control. The data indicate that the alligator serum exhibits potent anti-HIV-1_{IIIB} activity at 20–64-fold dilutions (5 and 1.6% serum, Fig. 1A), with 100 and 89% reduction in CPE, respectively. The cell toxicity index at these serum concentrations was 0, as 100% cell viability was observed in the cultured CEM-SS cells treated with 5.0 and 1.6% alligator serum (Fig. 1B). The anti-HIV-1_{IIIB} activity of the serum is difficult to assess at concentrations above 5% due to the inherent toxicity to the CEM-SS cells (Fig. 1B). However, it is likely that both the anti-HIV-1_{IIIB} activity and the CEM-SS cell toxicity are mediated by the serum complement system, as heat treatment (56 °C, 30 min) of the serum obliterated both effects (Fig. 1A and B). Like human complement activity, alligator serum complement is sensitive to heat inactivation (Merchant et al., 2004). The IC_{50} is defined as the serum concentration that inhibits 50% of viral activity, while the TC_{50} is defined as the serum concentration that reduces the mammalian cell culture viability to 50%. The IC_{50} value for HIV-1_{IIIB} antiviral activity was determined to be 0.85% serum, while the TC_{50} for CEM-SS cell toxicity was 32.8% serum. In contrast, both the IC_{50} and TC_{50} values were >50% serum for the heat-treated serum. AZT was included in the assay as a positive control antiviral compound (IC_{50} = 6.1 nM; TC_{50} > 1000 nM).

The anti-West Nile virus (WNV) activity of alligator serum was investigated by employing a cell-based assay in which Vero African green monkey kidney cells were infected with the WNV strain NY-99. The assays were conducted as previously described (Anderson and Rahal, 2002). Similar to the HIV-1 assay, the results represent the means of three independent determinations for each serum dilution and are expressed as % reduction in CPE. Ribavirin (IC_{50} = 80.6 μg/mL; TC_{50} > 200 μg/mL) was included as a positive control antiviral compound. The data reveal that the alligator serum shows moderately high activity against WNV at relatively low dilution (Fig. 2A). For instance, 16% alligator serum exhibited 77% of maximal anti-WNV activity. In addition, this same serum dilution produced a 24% reduction in Vero cell viability due to its cell toxicity (Fig. 2B). Higher serum concentrations (50% serum) reduced cell viability by 60%, while the anti-WNV effect was reduced to 32%. The full potential of the anti-WNV properties of alligator serum are difficult to evaluate due to the toxicity of the serum toward the Vero cells. An IC_{50} value for WNV antiviral activity was determined to be 4.3% serum, while the TC_{50} for Vero cell toxicity was 36.3% serum. Moreover, it is interesting to note that, unlike the anti-HIV effects, the anti-WNV effects and the Vero cell toxicity were virtually identical in untreated and heat-treated alligator serum (Fig. 2A and B). These data indicate that the anti-WNV activity and Vero cell toxicity

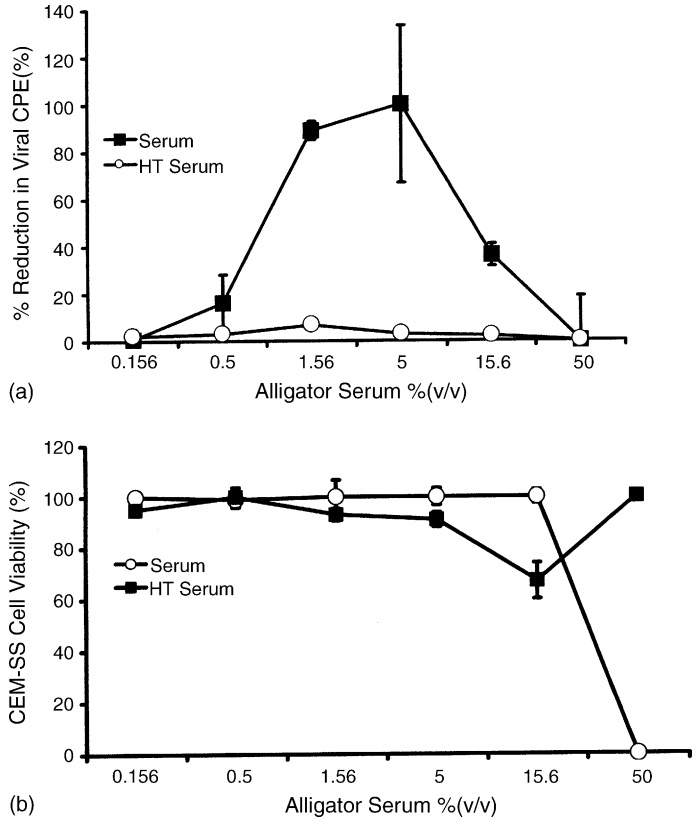

Fig. 1. Anti-HIV-1_{IIIB} activity and CEM-SS cell toxicity of alligator serum. (A) Anti-HIV-1_{IIIB} activity expressed as % reduction in viral CPE. (B) CEM-SS cell toxicity expressed as % cell viability. HT: heat-treated.

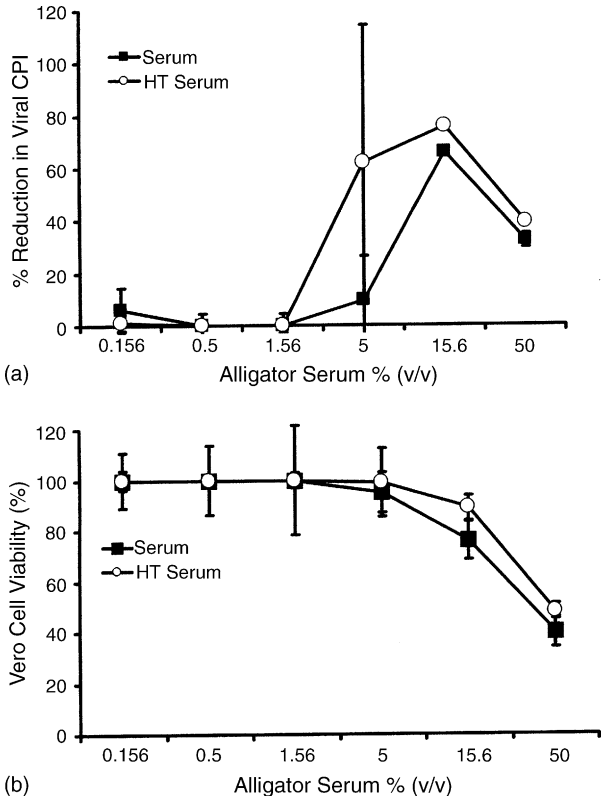

Fig. 2. Anti-WNV activity and Vero cell toxicity of alligator serum. (A) Anti-WNV activity expressed as % reduction in viral CPE. (B) Vero cell toxicity expressed as % cell viability. HT: heat-treated.

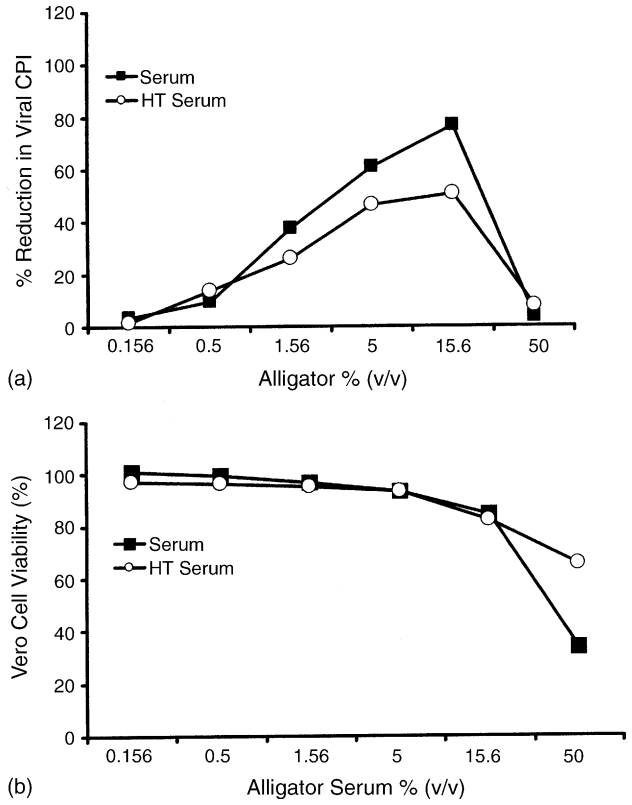

Fig. 3. Anti-HSV-1 activity and Vero cell toxicity of alligator serum. (A) Anti-HSV-1 activity expressed as % reduction in viral CPE. (B) Vero cell toxicity expressed as % cell viability. HT: heat-treated.

are not mediated by the heat-sensitive serum complement protein system. These activities may be due to the expression of cationic peptides, interferon-like molecules, or some other heat stable molecule with antiviral activity.

The activity of alligator serum against herpes simplex virus-type 1 (HSV-1) was tested via a cell-based assay in which Vero cells were infected with HSV-1 strain HF as previously described (Buckwold et al., 2004). Again, the results represent the means of three independent determinations for each serum dilution and are expressed as % reduction in viral CPE. Acyclovir (IC_{50} = 7.1 μM; TC_{50} > 100 μM) was included as a positive control antiviral compound. Alligator serum exhibited moderate antiviral activity against HSV-1. Treatment of HSV-1 with six-fold and 20-fold dilutions of alligator serum afforded the Vero cells 76 and 61% protection relative to the virus control (16 and 5% serum, Fig. 3A). In addition, treatment of the Vero cells with these concentrations of serum resulted in only modest reductions in cell viability (15 and 7%, respectively; Fig. 3B). The IC_{50} value for HSV-1 antiviral activity was determined to be 3.4% serum, while the TC_{50} for Vero cell toxicity of 39.1% serum confirmed the Vero cell toxicity observed in the WNV assay. The activity was not substantially lower in the heat-treated than in the non-treated serum ($p > 0.05$). Likewise, the cytotoxic sensitivity of the Vero cells to the heat-treated serum is not significantly different than the non-treated serum ($p > 0.05$, Fig. 3B), but the cytotoxic effect is not completely suppressed as the heat-treated serum elicited an 18% reduction in Vero cell CPE. These data illustrate that the mechanism of the anti-HSV-1 activity of alligator serum is not mediated by the serum complement system.

It is interesting to note that vertebrates as ancient as alligators display natural innate immunity to viruses that they in all likelihood have not regularly encountered during their evolution. The fact that alligator serum was least effective against the West Nile virus is supported by reports of isolated West Nile infections in alligator populations (Malakoff, 2003; Miller et al., 2003). The anti-HIV-1 data presented in this study, along with the wide spectrum of antibacterial and antiparasitic properties of alligator serum (Merchant et al., 2003, 2004), suggest that alligators have evolved a strong and broad-acting serum complement system. These data are consistent with the hypothesis that some phylogenetically ancient vertebrates, such as certain reptiles and teleost fish, have evolved a more complex innate immune system en lieu of adaptive immunity (Sunyer et al., 1997). However, the activities against the HSV-1 and WNV show that other antiviral mechanisms are active in alligator serum. To our knowledge, this is the first report of antiviral activities against enveloped viruses in the serum of lower vertebrates.

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