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Suppression of Respiratory Syncytial Virus Infection in Cotton Rats by Bis(5-Amidino-2-Benzimidazolyl)Methane

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Intraperitoneal administration of bis(5-amidino-2-benzimidazolyl)methane at well-tolerated daily doses of 25 mg/kg subsequent to challenge and for 3 days thereafter effected over a 1-log reduction in the amount of virus recovered from lungs of cotton rats inoculated intratracheally with respiratory syncytial virus. When animals were immunosuppressed to prolong virus shedding, the reduction in recovered virus achieved with a 7-day dosing schedule of bis(5-amidino-2-benzimidazolyl)methane exceeded 2 logs.

In recent years we have demonstrated that certain aromatic amidino compounds can block cytopathological changes in respiratory syncytial virus (RSV)-infected cell cultures (1–3, 12). The cells remain viable, and formation of the characteristic syncytial aggregates fails to take place. The antiviral effect of these compounds is not based on an inhibition of virus replication per se, but appears to be the result of interference with virus-cell membrane interactions. If the inhibitor is added to the cell cultures at the same time as the inoculum, then virus penetration is blocked. If the inhibitor is added after penetration has occurred, then newly formed virions seem to be unable to enter other cells or to induce linkage of the host cell with adjacent cells in a fusion process (2). Under either condition, multiple-cycle yields of the virus are significantly reduced.

These encouraging results obtained with amidino compounds against RSV in vitro led us to extend our studies to the in vivo situation and to evaluate the potential therapeutic use of the most effective in vitro agent. The results, as reported in this communication, show the effect of treatment of RSV-infected cotton rats with the diamidino compound bis(5-amidino-2-benzimidazolyl)methane (BABIM).

The Long strain of RSV (ATCC VR-26) was used in this study. The virus was grown in suspensions of HEp-2 cells and, after virus absorption, the infected cells were seeded in 75-cm^2 plastic culture flasks containing minimal essential medium plus 10% fetal bovine serum. When viral cytopathic effects were maximal (72 to 96 h), the contents were frozen at −70°C. Typical lysates contained 10^7 to 10^8 50% tissue culture infective doses per ml. Virus yields were quantitated by calculating 50% endpoints as previously described (9).

BABIM was synthesized by us according to a previously reported method (11). The purity of the compound was determined by nuclear magnetic resonance spectrometry and elemental analysis (Galbraith Laboratories, Inc., Knoxville, Tenn.). The compound was tested as the dihydrate of the tetrahydrochloride salt.

Cotton rats (*Sigmodon hispidus*) were obtained from the breeding colony at the University of North Carolina at Chapel Hill. This colony was established from breeding pairs provided through the courtesy of R. M. Chanock at the National Institutes of Health, Bethesda, Md. In all drug effectiveness studies, cotton rats between 8 to 10 weeks of age, between 60 to 120 g in weight, and with equal distribution of sexes between control and treated populations were used. The toxicity studies of BABIM were carried out on slightly older (10 to 16 weeks) and heavier (80 to 160 g) animals.

Treatment of immunocompetent RSV-infected animals was carried out as follows. Twenty cotton rats were weighed and then anesthetized by a combination of 60 mg of ketamine (intramuscular) and 21 mg of pentobarbital sodium (intraperitoneal) per kg of body weight. The anesthetized animals were given RSV (10^7 50% tissue culture infective doses per ml) by intratracheal inoculation (0.1 ml) (4). While still under anesthesia (5 min after challenge), 10 rats were given intraperitoneal injections of 25 mg of BABIM per kg dissolved in sterile phosphate-buffered saline (PBS), and 10 were given PBS solution only. On the following 3 days, each rat was weighed and received a daily dose of either BABIM or PBS solution. On day 5 of the experiment, all animals were sacrificed by lethal intraperitoneal injection of 32.5 mg of pentobarbital sodium, and the lungs were removed. The right lung was homogenized in sterile sand and 10% minimal essential medium, and titers of RSV were determined; the left lung was prepared for histological examination.

Treatment of immunosuppressed RSV-infected animals was carried out as follows. Twenty-four cotton rats were immunosuppressed with intraperitoneal injections of 100 mg of cyclophosphamide per kg on day 1 of the experiment, and the surviving animals were given the same dose again on day 4 (7). Previous studies have shown that a single dose of cyclophosphamide effects leukocyte suppression of 5 days' duration. On day 2, 14 rats (12 cyclophosphamide treated and 2 untreated) received intraperitoneal doses of 25 mg of BABIM per kg dissolved in PBS, and the remaining 14 animals (12 cyclophosphamide treated and 2 untreated) received an injection of PBS solution only. Within 2 h of treatment, all of the animals were weighed, anesthetized (four control cyclophosphamide-treated animals and one BABIM-treated animal did not recover from ether anesthesia), and exposed to RSV (10^7 50% tissue culture infective doses per ml) by intratracheal inoculation (0.1 ml). On each of the following 6 days, the animals were weighed and received their respective injections of either BABIM or PBS solution. On day 9 of the experiment, all of the animals were

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TABLE 1. Treatment of RSV-Infected Cotton Rats with BABIM

No. of animals	Cyclophosphamide treatment	No. of days of treatment with BABIM[a]	Day of sacrifice after challenge with RSV	Mean ± SD lung titers (log $TCID_{50}$)[b]
10	−	0	5	5.06 ± 0.50
10	−	4	5	3.79 ± 0.32
8	+	0	8	5.72 ± 0.65
11	+	7	8	3.25 ± 0.37
2	−	0	8	0
2	−	7	8	0

[a] BABIM was given intraperitoneally at a dose of 25 mg/kg.
[b] $TCID_{50}$, 50% Tissue culture infective dose (determined per gram of lung tissue). $P < 0.001$ by the Student t test.

sacrificed by lethal injection, and the lung tissue was analyzed for RSV and histological changes.

Tissue was studied from uninfected animals, RSV-infected animals, and BABIM-treated, RSV-infected animals. Samples fixed in 10% buffered Formalin for 48 h, dehydrated in serial alcohol solutions, and embedded in paraffin were sectioned and stained with hematoxylin and eosin. Histological sections were examined by light microscopy for inflammatory infiltrates (peribronchial, peribronchiolar, alveolar septal, and perivascular) and for changes in the bronchiolar epithelium (hyperplasia, giant cells, necrosis, and cytoplasmic inclusions).

For acute toxicity testing, BABIM was administered intraperitoneally to groups containing at least 10 cotton rats per group at five dose levels (50, 75, 100, 125, and 150 mg of BABIM per kg). By definition, only deaths occurring within the first 24 h after injection were used in the determinations of the 50% lethal dose. Still, no deaths after the 24-h period were noted. The dose-response data were evaluated by the Litchfield-Wilcoxon method (8) to give the 50% lethal dose, the slope of the dose-response curve, and the 95% confidence limits. Also, a group of 10 animals were subjected to prolonged administration of the drug to determine the effect of accumulated doses. In the prolonged dosing experiment, the animals were given 30 mg of BABIM per kg by intraperitoneal injection daily for 7 days. The animals were checked daily, and any toxic sypmtoms, such as anorexia, lethargy, and weight loss, were recorded.

The effect of BABIM treatment on the virus titers from the lungs of RSV-infected cotton rats is shown in Table 1. Immunocompetent cotton rats receiving daily intraperitoneal injections of BABIM (25 mg/kg) for 4 days had statistically lower mean lung RSV titers than those receiving injections of PBS only. The effect of the drug was more pronounced when treatment could be extended to seven daily doses, as was done with the immunosuppressed animals. In this case, the BABIM-treated animals showed a drop of 2.45 logs in mean lung virus titers as compared with the untreated animals. In the four animals not immunosuppressed with cyclophosphamide (two BABIM treated and two untreated), no virus was recovered on day 8 after challenge.

Microscopic examination of lung tissue showed that there were only very minor histological changes in treated and control animals. Most animals in these two experiments had no detectable pulmonary lesions, and animals that did show some histological changes fell in no group which could be classified according to virus titers, immunosuppression, or BABIM treatment.

The acute 50% lethal dose of BABIM for cotton rats was found to be 130 mg/kg by intraperitoneal injection, with a 95% confidence level of 118 to 148 mg/kg. Animals receiving daily injections of 30 mg of BABIM per kg for 7 days showed some evidence of weight loss, anorexia, and lethargy. However, the symptoms were not severe, and no deaths were recorded at this dose level. In addition, all animals recovered completely when the drug was withdrawn. These observations suggest that 25 mg of BABIM per kg is the maximum dose level that can be used in the sustained treatment of infected cotton rats.

This investigation clearly demonstrates the ability of BABIM to lower the amount of virus recovered from the lungs of RSV-infected cotton rats. Daily intraperitoneal injections of BABIM for 4 days produced greater than 90% reduction in virus titers from lung tissue of immunocompetent cotton rats, whereas a greater than 99% reduction in virus content was observed in immunosuppressed animals treated for 7 days. This reduction was not due to carry-over of BABIM with tissue into the lung suspension titrations; infected suspensions in which the virus was heat inactivated had no effect on the titer of stock virus.

The relationship between this degree of reduction in virus titers and the therapeutic potential of BABIM is speculative. However, a comparison can be made with the effect of a similar reduction in virus titers produced by ribavirin in animals experimentally infected with influenza virus. In that instance, a reduction in virus titers of the magnitude seen with BABIM against RSV resulted in a significant reduction of mortality and of pulmonary inflammation (6, 14).

Other investigators have achieved control of RSV infection by the use of ribavirin (5, 6, 10) or the injection of neutralizing monoclonal antibodies to RSV glycoproteins (13). Compared with a reported experiment with ribavirin, BABIM appears to be more effective against the infection in cotton rats when the intraperitoneal route of administration is chosen. At this point it remains undetermined, however, whether this superiority would pertain to the aerosol mode of application. Such inhalation therapy has already proven moderately successful with ribavirin not only in the treatment of cotton rats but also of RSV-infected children (10). Further studies are in progress to assess the usefulness of BABIM in an aerosol regimen and to obtain complete pharmacokinetics and optimal dose schedules.

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