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Activity against Rhinoviruses, Toxicity, and Delivery in Aerosol of Enviroxime in Liposomes

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Enviroxime has been shown to inhibit the replication of rhinoviruses and other enteroviruses in concentrations as low as nanograms per milliliter in in vitro assays but is markedly less effective in clinical trials. The marked hydrophobicity and water insolubility of this compound may be a factor for this disparity. To overcome this handicap, we incorporated enviroxime into liposomes and then tested the antirhinovirus activity and toxicity of the liposome-incorporated enviroxime (LE) in cell culture and studied its administration by small-particle aerosol. Free enviroxime and LE were found to have equivalent efficacies against rhinovirus strains 1A and 13 in in vitro assays; however, preparations of LE were 10- to ≥50-fold less toxic to tissue culture cells than was free enviroxime. In contrast to free enviroxime, which could not be delivered by small-particle aerosol because of its water insolubility, LE (4 mg/ml) was readily and successfully delivered by small-particle aerosol to the upper and lower respiratory tracts of mice; after just 20 min, significant levels of enviroxime were detected in the lungs and noses of exposed mice. Moreover, mice exposed to aerosols of liposomes containing both enviroxime and fluorescein isothiophosphatidylethanolamine showed accumulations of the fluorescent marker in the lungs, particularly in or around the tall columnar epithelial cells lining the bronchi and bronchioles.

Enviroxime, 2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime, is reportedly a highly active inhibitor of picornavirus replication in tissue and organ cultures (2; D. C. DeLong, J. D. Nelson, C. Y. E. Wu, B. Warren, J. Wikel, J. Chamberlin, D. Montgomery, and C. J. Paget, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S128, p. 234). In contrast, the reported efficacy of this drug in preventing or treating human rhinovirus infections in field trials has been quite variable. Thus, although Phillpotts et al. found a significant reduction in disease and virus shedding in a prophylactic challenge study (19) and some improvement in illness in a therapeutic trial (20), two groups, Hayden and Gwaltney (5) and Miller et al. (16), failed to see significant reductions in illness or infection in prophylactic or therapeutic studies. Although numerous factors, such as the irritation caused by the carrier and the uneven distribution of the drug in the respiratory tract, have been suggested as causing the disparate results obtained in these tests (9, 16), the primary underlying factor may be the relative insolubility of enviroxime in water or water-based media.

Much higher concentrations of enviroxime can be incorporated into liposomes (B. E. Gilbert, H. R. Six, S. Z. Wilson, P. R. Wyde, and V. Knight, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 986, 1987), lipid bilayers which have been used in numerous studies to deliver antimicrobial agents (1, 3, 6, 8, 10–12, 23). In addition to increasing the solubility of enviroxime, incorporation into liposomes may also increase the therapeutic index of this compound. Liposomes have been shown to increase the therapeutic indices of a number of drugs. These increases occurred as a result of decreased toxicity (e.g., amphotericin and streptomycin [10–12]), more efficient targeting (e.g., directed delivery of antiviral compound to cells of the reticuloendothelial systems [1, 3, 6, 8, 23]), or a delay in the clearance of the drug from the body (10–12).

Incorporation of enviroxime into liposomes will also allow the drug to be delivered by small-particle aerosol (SPA). SPAs containing antiviral substances have been used with particular success against respiratory virus infections (4, 7, 13, 14, 18, 22, 24). The primary advantage of this mode of delivery is that the antiviral particles generated deposit rather uniformly over the surfaces of the respiratory tract, leading to local levels of the delivered antiviral agent that may exceed the levels achieved by parenteral or oral administration (22, 25).

In the present report, we describe findings comparing the antiviral efficacies of free enviroxime and enviroxime incorporated into liposomes (LE) on rhinovirus replication in human KB carcinoma cells and the toxicity of these substances on several tissue culture cell lines. We further report on the delivery of LE to mice by SPA and the detection of the delivered drug in the respiratory tract by using a high-performance liquid chromatography (HPLC) assay. Evidence is provided that suggests the localization of liposome contents in the pulmonary epithelium.

MATERIALS AND METHODS

Mice. Swiss mice (6 to 10 weeks old) from the Animal Genetics and Production Branch, National Cancer Institute, Bethesda, Md., were used in all experiments. These mice were housed in cages covered with barrier filters and were fed with mouse chow and water ad libitum.

Viruses. Rhinovirus strains 1A (RV1A) and 13 (RV13) were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). Stocks of these viruses were prepared by inoculating flasks of KB tissue culture cells with seed virus, incubating the infected flasks at 33°C, and waiting until the monolayers exhibited more than 90% cytopathic effects (CPE). At that time, the overlying medium was collected, pooled, and centrifuged to remove cellular debris. The resulting supernatant was removed, portioned, and stored at −70°C. The mean titers of the RV1A and RV13

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virus stocks in KB cells were 5×10^6 and 2×10^7 PFU/ml, respectively.

Tissue culture. Starting cultures of mouse L929 (ATCC CCL1), HeLa (ATCC CCL2), Madin-Darby canine kidney (MDCK; ATCC CCL34), and KB (ATCC CCL17) tissue culture cells were obtained from the ATCC. These cultures were routinely serially passaged when confluent by using Eagle minimal essential medium (catalog no. 51-41278; Hazleton Research Products) supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.2% sodium bicarbonate, and 10% fetal calf serum (10% FCS-MEM).

Liposomes. Multilamellar liposomes were prepared from dried lipid films of egg yolk phosphatidylcholine (EYPC). Enviroxime or fluorescein isothiophosphatidylethanolamine (FL-PE) was added directly to EYPC-chloroform solution. The lipid film was then dissolved in tertiary butyl alcohol, and the solvent was removed by rotary evaporation (Rotavapor apparatus; Brinkmann Instruments, Westbury, N.Y.). This process was repeated a second time. The lipid films were then placed in a dessicator for at least 1 h under vacuum. The resulting films were dispersed in sterile phosphate-buffered saline by vortexing. The final concentration of EYPC in these preparations was 15 mg/ml. Control liposomes were prepared similarly, but without enviroxime.

Aerosol delivery. A nebulizer (model 1920; Puritan Bennett Co., Los Angeles, Calif.) was used to generate continuous SPAs of liposomes or LE. This nebulizer has a single air jet and a 250-ml reservoir. When operated at 26 lb/in^2, it produces an aerosol of liposomes containing particles with an aerodynamic mass median diameter of 2.5 ± 3.0 μm at a flow rate of 13 to 15 liters/min (Gilbert et al., 27th ICAAC). In these experiments, each preparation was diluted in 50 ml of sterile distilled water (catalog no. 670-5230; GIBCO Laboratories, Grand Island, N.Y.), placed in a delivery reservoir, and administered for up to 120 min. In early experiments, a control group of mice which were exposed to SPAs of empty liposomes was included in each experiment. However, as no antiviral effect was ever detected in the lungs of these mice, such controls were used only intermittently thereafter. The fluid remaining in each delivery reservoir at the end of each aerosol treatment was checked for drug concentration and microbial sterility.

Collection of lung fluids and nose washes. At selected intervals, mice were killed by cervical dislocation, and the lungs of each animal were collected. The mice were then decapitated, and the lower jaw was removed from each head. Each lung was transpleurally lavaged by using 3 ml of RPMI 1640 medium (catalog no. 320-1875; GIBCO), as described previously (27); nose washes were collected by pushing 1 ml of RPMI 1640 medium through each nostril and capturing the effluent from the posterior opening. Lung fluids and nose washes were then assessed for antiviral activity as described below.

In experiments involving mice exposed to FL-PE, lungs were removed, rinsed in saline, and then embedded in OCT compound (Tissue-TeK; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.), with liquid nitrogen immersion used to freeze the OCT compound. Thin sections of lung tissue were made by using a cryostat and were mounted on glass slides. These sections were observed for fluorescence as described below (Histological methods).

HPLC quantification of enviroxime and LE. Enviroxime was quantified by HPLC with monitoring at 215 nm (Waters Associates, Inc., Milford, Mass.). All measurements were made at ambient temperature on a stainless steel HPLC column (Microsorb C18; particle size, 5 μm; length, 25 cm; inner diameter, 4.6 mm; Rainin Instrument Co., Emeryville, Calif.). The mobile-phase solvent was acetonitrile-water (60:40) flowing at a rate of 1 ml/min. Under these conditions, enviroxime eluted at around 4 min. This assay system was capable of detecting an enviroxime standard at 5 ng/10-μl injection volume and had a linear response up to at least 500 ng/10 μl-injection volume.

Because of the phosphatidylcholine component of the liposomes, it was necessary to separate the enviroxime from the phospholipid before HPLC analysis to prevent binding of the lipid to the C18 column. Separation was accomplished with the use of Sep-Pak C18 cartridges for rapid sample preparation (Waters). Sep-Pak cartridges were activated by the sequential addition of 10 ml each of absolute methanol, 100% acetonitrile, and water. Enviroxime was extracted from the liposomes by using Sep-Pak cartridges and the following procedure. (i) A total of 1 to 2 ml of liposomes in aqueous solution was added to an activated Sep-Pak cartridge, and the eluate (fraction 1) was discarded; (ii) the Sep-Pak cartridge was washed with 4 ml of water, and the eluate (fraction 2) was discarded; (iii) each cartridge was washed with 0.5 ml of acetonitrile-water (80:20), and the eluate (fraction 3) was discarded; and (iv) enviroxime (fraction 4) was eluted from the Sep-Pak cartridge with 2.0 ml of acetonitrile-water (80:20). The efficacy of separation and percent recovery of enviroxime from liposome preparations were determined by adding trace amounts of [^{14}C]enviroxime (ca. 3.5×10^5 cpm; 13.8 mCi/mmol [89.7% pure]; 17.8 pmol/cpm) and [^{3}H]phosphatidylcholine (3.7×10^5 cpm; 57 Ci/mmol; 42.5 pmol/cpm) prior to liposome preparation and evaluating the amount of radioactivity in each of the fractions.

Enviroxime levels in mouse lung lavages, nasal wash fluids, and sera were determined by using the Sep-Pak and HPLC methodology described above.

Biologic measurement of antiviral activity. Biologic assays for enviroxime activity were performed in 96-well flat-bottom plates (catalog no. 76-003-05; Flow Laboratories, Inc., McLean, Va.). All dilutions and tissue culture suspensions were prepared with 5% FCS-MEM. Briefly, 0.05 ml of test fluid was added in duplicate or quadruplicate to the first wells of the test plates and diluted by serial twofold dilutions. Approximately 100 median tissue culture infective doses of RV13 or RV1A in 0.05 ml was then added to each well, except for tissue culture control wells, followed by the addition of KB tissue culture cells (approximately 2×10^4 cells in 0.1 ml). Tissue control wells containing medium and cells (no virus or antiviral compound) and virus control wells containing medium, virus, and KB cells (no antiviral compound) were included in each assay. For each assay, each challenge virus was back titrated. All plates were incubated at 33°C in a 5% CO_2 incubator. The virus control wells in each plate were observed daily. When CPE in these wells were 80 to 100% evident, the degree of CPE in all wells was recorded. The minimal inhibitory dose was then determined by calculating the mean concentration of test substance in the last wells of replicate rows that inhibited virus CPE by ≥50% as compared with the CPE in virus controls.

In several experiments, antiviral activity was also assessed by a plaque reduction assay. In these assays, monolayers of KB cells were adsorbed with 100 to 200 PFU of RV1A or RV13 and then overlaid with agar containing serial dilutions of test material. Controls for this assay consisted of virus controls (no antiviral substance added to the overlay), drug controls (drug but no virus added to the monolayers),

tissue controls (no virus or drug in overlay), and a back titration of virus used in each assay. The plates were incubated at 33°C in a 5% CO_2 incubator for 4 days. At that time, the monolayers in each well were fixed with Formalin and then stained with crystal violet. PFU were enumerated, and titers were expressed as the minimal concentration of antiviral substance which reduced plaque formation by 50% or more as compared with the mean number of plaques in the virus control.

Toxicity tests. Assessment of the toxicity of different antiviral preparations for mouse L929, MDCK, and human KB and HeLa tissue culture cells was performed in 96-well flat-bottom plates. Briefly, 0.05 ml of liposomes (15 mg of EYPC per ml), 95% methanol, free enviroxime (1 to 4 mg/ml) in 95% methanol, or LE (1 to 4 mg of enviroxime per ml in liposomes containing 15 mg of EYPC per ml) was added to the first wells of test plates, using four rows per preparation, and diluted by serial twofold dilutions. (Methanol was required as a carrier, since enviroxime in concentrations of >2 μg/ml was not soluble in water or in water-based medium.) Additional control wells containing 5% FCS-MEM only were also included in each assay. A 0.1-ml volume of the appropriate tissue culture cells (2×10^4 cells) was then added to each well. Plates were incubated at 33°C in a 5% CO_2 incubator. After 4 days, the fluid from each well was removed. The monolayers were then fixed with Formalin, stained with 2% crystal violet in 95% ethanol, and observed for CPE and confluency. The test material was considered toxic if the monolayer observed in a well exhibited ≥50% CPE. Toxicity was expressed as the mean minimal toxic concentration and was determined by calculating the mean concentration of antiviral substance or carrier in the last wells that exhibited ≥50% CPE.

Histological methods. Sections to be stained with hematoxylin and eosin were fixed in buffered Formalin, embedded in low-melting-point paraffin, and sectioned to 5-μm thickness. Those sections of lungs exposed to liposomes containing both enviroxime and FL-PE were sectioned on a cryostat and kept at −20°C until observed for fluorescence. All fluorescence studies were made with a Leitz Wetzlar Ortholux II UV microscope equipped with an HBO-200W mercury burner, a BG12 excitation filter, and a 470 barrier filter.

Statistics. Means, standard deviations, and statistical significance by Student's *t* test were determined for different samples, as described by Sokal and Rohlf (21). All data were transformed (log_2 or log_3) before statistical analysis and converted back to whole numbers for presentation.

RESULTS

Enviroxime and LE toxicity. A comparison of the toxicity of methanol, enviroxime, and LE for mouse L929, MDCK, and human KB and HeLa tissue culture cell lines is shown in Table 1. In these tests no CPE were evident in wells containing empty liposomes, whichever cell line was used. These tests included wells containing the highest concentration of liposomes tested (final concentration, 3 mg of EYPC per ml). In contrast, both methanol (the carrier used to dissolve free enviroxime) and enviroxime in methanol caused CPE in all tissue culture cell lines tested. The mean concentration of methanol that induced ≥50% CPE in the different cells (shown in parentheses in Table 1) varied and ranged from 1.0 to 12%. The mean toxic concentrations of enviroxime in methanol that induced ≥50% CPE in the different tissue cells similarly varied but were consistently lower than those of methanol alone; values for the methanol component ranged from 0.1 to 3%, and values for the enviroxime component ranged from 1 to 31 μg/ml. Except for the mean values obtained in experiment 1 with HeLa cells, all means obtained (for a given tissue) for methanol or enviroxime in methanol were statistically different when compared in Student's *t* test ($P \leq 0.05$). The lower mean toxic concentrations of enviroxime in methanol for each tissue culture cell line indicated the greater toxicity of enviroxime in methanol compared with methanol alone. Regardless, no toxicity was observed with LE, even in those wells containing the highest concentrations of LE tested (final concentrations of 125 μg/ml for enviroxime and 3 mg/ml for EYPC).

TABLE 1. Comparison of mean concentrations of enviroxime and LE that caused ≥50% CPE in tissue culture cell lines[a]

Prepn	Expt no.	Mean minimal toxic concn of enviroxime (μg/ml) (mean minimal toxic concn of methanol [%])[b]			
		KB	HeLa	L929	MDCK
Liposomes only	1	NCPE[c]	NCPE	NCPE	NCPE
	2	NCPE	NCPE	NCPE	NCPE
Methanol only	1	(1)	**(1)**	(3)	(3)
	2	(3)	(12)	(6)	(12)
Enviroxime in methanol	1	2 (0.1)	5 **(0.5)**	4 (0.4)	8 (0.7)
	2	8 (0.7)	31 (3.0)	1 (0.1)	8 (0.7)
LE	1	>125	>125	>125	>125
	2	>125	>125	>125	>125

[a] Final observations were made 4 days after culture was initiated.
[b] Each test was performed in quadruplicate. Values in boldface were not statistically different from each other when compared by using Student's *t* test; in all other instances, for a given tissue, the mean toxic concentrations of methanol, enviroxime in methanol, and LE were statistically different ($P \leq 0.05$).
[c] NCPE, No CPE evident even at maximum concentration tested (final concentration, 3 mg of EYPC per ml).

In vitro efficacy of enviroxime and LE. Despite differences in their relative toxicity, enviroxime and LE were equally active in inhibiting RV13 or RV1A replication in KB tissue culture cells (data not shown). Both enviroxime and LE reduced plaque formation by ≥50% compared with virus controls at a final concentration of 0.04 μg/ml and totally inhibited plaque formation at a final concentration of 0.4 μg/ml. In tissue culture assays using CPE as the endpoint, both enviroxime and LE had virtually identical values (range of MICs depending on the amount of challenge virus used, 0.03 to 0.09 μg/ml for enviroxime and 0.02 to 0.06 μg/ml for LE). In contrast, liposomes lacking drug had no inhibitory effect in either assay, even at the maximum concentration tested (final concentration, 6 mg of EYPC per ml).

Delivery of LE to lungs. Some fluorescence was evident in cryostat sections prepared from lungs of mice exposed for 10 min to SPAs of liposomes containing both enviroxime and FL-PE (Fig. 1). Greater accumulations of fluorescence in the large and small passageways were evident by 30 min (results not shown), and this accumulation increased continually with time. Figure 1B shows a representative section taken from lungs collected 120 min after the start of aerosolization. There was an accumulation of fluorescence, particularly in or around the tall columnar epithelium lining the bronchioles. At all time intervals (up to 120 min), fluorescence in the parenchymal tissues of exposed mice was minimal, while sections of lungs from mice not exposed to FL-PE-containing liposomes did not fluoresce (results not shown).

Histologic evaluations. No notable histopathologic findings were observed in sections of lungs taken 24 to 72 h after

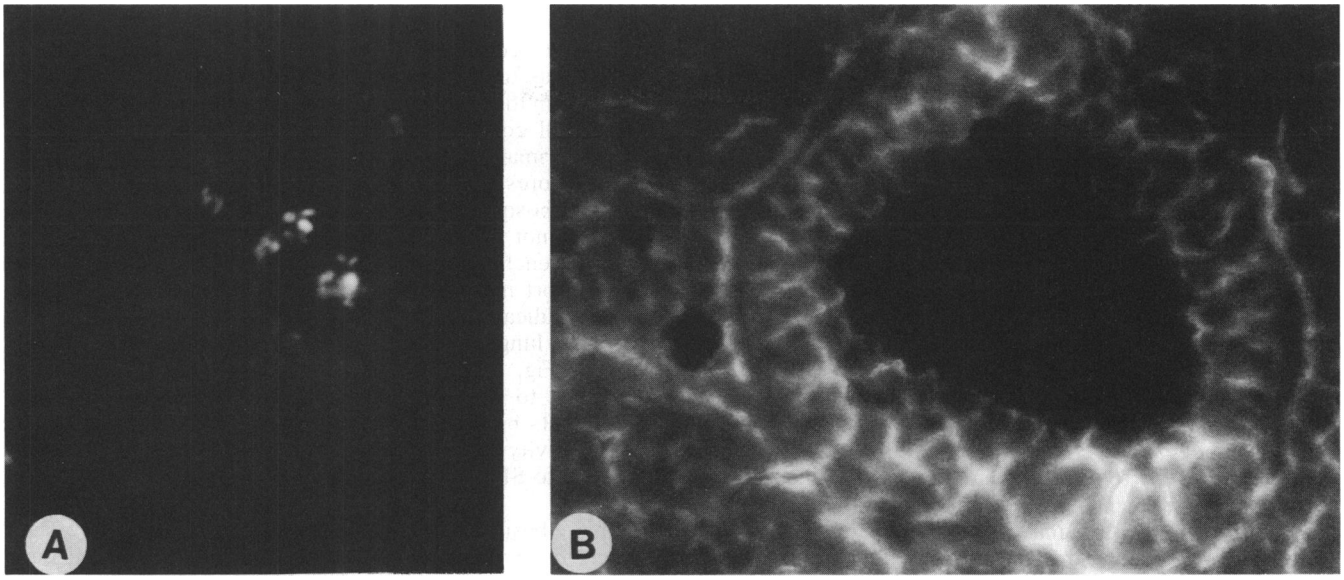

FIG. 1. Photomicrographs of sections of a lung from a mouse exposed for 10 min (A) or 120 min (B) to SPAs of liposomes containing enviroxime and FL-PE (magnification, ×500).

aerosolization ceased, whether the lungs were from normal mice, mice exposed for 2 h to continuous SPAs of empty liposomes, or mice exposed for 2 h to continuous SPAs of LE (4 mg of enviroxime per ml). All observations were made by trained pathologists and were done blind.

Levels of enviroxime in respiratory tract. Figure 2 compares enviroxime levels in lung lavage and nose wash fluids from mice exposed to continuous SPAs of LE for 20 min. Significant levels of drug were present in nose washes of test mice immediately after termination of aerosolization (0.65 ± 0.4 [mean ± standard deviation] μg per nose wash), 0.5 h later (0.69 ± 0.4 μg per nose wash), and at 1 h postaerosolization (0.63 ± 0.6 μg per nose wash). Thereafter, the concentration of enviroxime declined rapidly, and the drug was undetectable by 2 h.

Levels of enviroxime in the lungs of these mice were lower than levels observed in the nose. Immediately after aerosolization ceased and at 30 min postaerosolization, 0.36 ± 0.1 μg of drug per lung was detected. However, 1 h after aerosolization ceased and thereafter drug was no longer detectable in the lung.

Figure 3 compares the antiviral activity of lung lavage

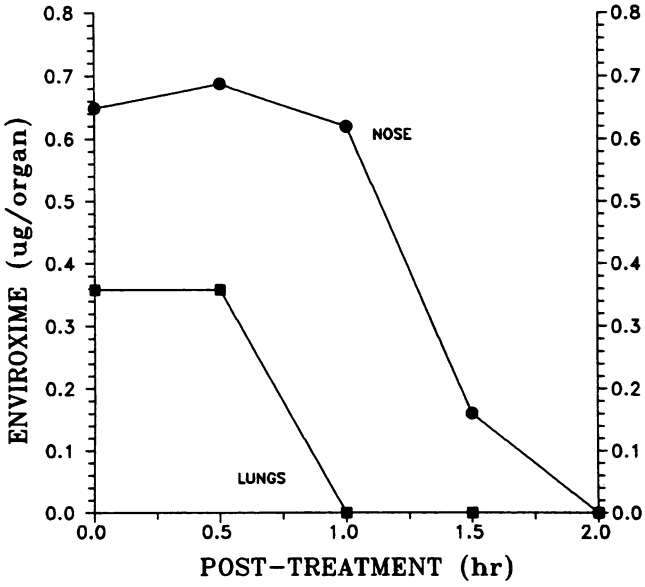

FIG. 2. Levels of enviroxime in nasal washes (●) and lungs (■) of mice exposed for 20 min to SPAs of LE (reservoir concentration, 4 mg/ml), as determined by HPLC.

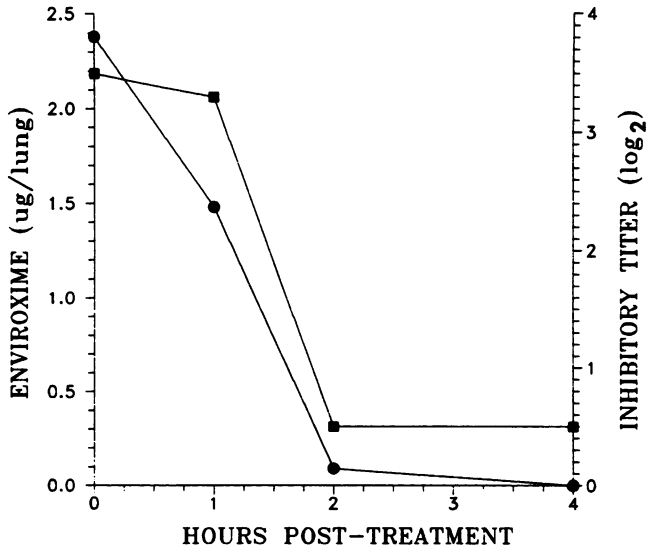

FIG. 3. Comparison of antiviral activity (log_2) with levels of enviroxime in lung lavage fluids obtained from lungs of mice exposed for 90 min to SPAs of LE (reservoir concentration, 4 mg/ml). ●, Mean enviroxime values as determined by HPLC; ■, geometric mean inhibitory titers (log_2) per 0.05 ml of lung lavage fluid. The latter test was performed in KB tissue cells, with approximately 33 median infective doses of RV13 as the challenge virus.

fluids obtained from lungs of mice exposed for 90 min to SPAs of LE with the levels of enviroxime as determined by HPLC. As indicated, antiviral activity was detectable for up to 2 h after aerosolization ceased and closely paralleled the levels of enviroxime in the lungs.

DISCUSSION

These studies were concerned primarily with the toxicity, antiviral efficacy, and delivery by SPA of liposomes containing the antirhinovirus compound enviroxime. The impetus to do the studies came from the fact that although free enviroxime is a potent inhibitor of rhinoviruses and other picornaviruses in vitro, the antiviral efficacy of enviroxime in clinical trials has been quite variable and disappointing. Several factors have been suggested as causes of the marked dichotomy of effects seen in the in vitro and in vivo studies of enviroxime (5, 9, 16). (i) Enviroxime or the vehicle used to deliver enviroxime to the nose may irritate the mucosa, and thus, efficacy may be masked; (ii) the natural rapid clearance of enviroxime in the nose may significantly reduce local drug concentrations; (iii) the drug may not be distributed equally throughout susceptible regions of the respiratory tract; and (iv) because of the insolubility of the drug, the concentrations of enviroxime delivered to the respiratory tract may be marginal or insufficient.

LE has a number of advantages over free enviroxime, and incorporation of the drug in liposomes may reduce or abrogate all of the problems listed above. First, significantly higher levels of drug can be put into liposomes compared with the small amount that can be dissolved in water-based medium or even in alcohol. In our study, enviroxime was only slightly soluble in water or in saline solution (no more than 2 μg/ml). Although as much as 1,000 μg of enviroxime per ml can be dissolved in 95% alcohol, this carrier is irritating to the linings of the respiratory tract and is not a practical carrier of a drug to be used for humans. In contrast, we have incorporated 6,000 μg of enviroxime per ml in liposomes.

Second, the use of liposomes to deliver a drug may decrease the toxicity of the drug. In a number of clinical trials, drugs that were known to be toxic in the free state were shown to be less toxic or nontoxic when given in liposomes (10, 11, 15, 23). In the present studies, enviroxime and the methanol carrier used to dissolve it both proved in in vitro tests to have some toxicity for mouse L929, MDCK, and human KB and HeLa cells (Table 1). However, when incorporated in liposomes, enviroxime, even at a final concentration of 125 μg/ml, proved nontoxic to all test cells. Despite the decreased toxicity, the antiviral efficacy of LE in in vitro tests was not altered; in assays using either plaque reduction or CPE reduction as the endpoint, enviroxime and LE preparations had equivalent MICs (between 0.02 to 0.04 μg/ml depending on the assay system and the amount of challenge virus).

A third advantage of putting enviroxime into liposomes is that LE, but not enviroxime, can be delivered to the respiratory tract by SPA. Significant levels of enviroxime were delivered to the upper and lower respiratory tracts when mice were exposed for just 20 min to SPAs of LE (Fig. 2). Delivery of other antiviral substances by SPA has been used with good success in treating respiratory virus diseases such as influenza and respiratory syncytial virus (4, 7, 13, 14, 17, 22, 24). There is some thought that when delivered by SPA antiviral substances effective against respiratory viruses may be more effective than parenterally administered drug (22, 26, 27).

Other advantages may also accrue. Liposomes have been shown to provide a depot effect (10, 11) and to target compounds to specific target cells (1, 3, 6, 8, 23). Results obtained in these studies suggest the targeting of enviroxime to the tall columnar epithelial cells lining the respiratory tract, a primary site of rhinovirus replication. Accumulation of the fluorescent marker FL-PE, codelivered with enviroxime in liposomes by SPA, occurred in or around these cells and was not particularly evident in or around alveolar or other parenchymal cells (Fig. 1). In addition, despite relatively short intervals of exposure (20 to 120 min), kinetic studies indicated the persistence of enviroxime in both the nose and lungs between 30 min to 2 h after aerosolization ceased (Fig. 2 and 3). Equally important, the enviroxime delivered to the respiratory tract in liposomes by SPA retained its biologic activity, as indicated by the antirhinovirus activity in lavage fluids obtained from lungs of mice exposed to SPAs of LE when tested in in vitro assays (Fig. 3).

On the basis of results obtained in these tests, future tests to determine the in vivo efficacy of LE delivered by SPA are planned.

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LITERATURE CITED

1. **Alving, C. R.** 1983. Delivery of liposome-encapsulated drugs to macrophages. Pharmacol. Ther. **22**:407–424.
2. **DeLong, D. C., and S. E. Reed.** 1980. Inhibition of rhinovirus replication in organ culture by a potential antiviral drug. J. Infect. Dis. **141**:87–91.
3. **Gregoriadis, G., and B. E. Ryman.** 1972. Lysosomal localization of α-fructofuranosidase-containing liposomes injected into rats. Some implications in the treatment of genetic disorders. Biochem. J. **129**:123–133.
4. **Hall, C. B., J. T. McBride, E. E. Walsh, D. M. Bell, C. L. Gala, S. Hildreth, L. G. TenEyck, and W. J. Hall.** 1983. Aerosolized ribavirin treatment of infants with respiratory syncytial virus infection. N. Engl. J. Med. **308**:1443–1447.
5. **Hayden, F. G., and J. M. Gwaltney, Jr.** 1982. Prophylactic activity of intranasal enviroxime against experimentally induced rhinovirus type 39 infection. Antimicrob. Agents Chemother. **21**:892–897.
6. **Kende, M., C. R. Alving, W. L. Rill, G. M. Swartz, Jr., and P. G. Canonico.** 1985. Enhanced efficacy of liposome-encapsulated ribavirin against Rift Valley fever virus infection in mice. Antimicrob. Agents Chemother. **27**:903–907.
7. **Knight, V., H. McClung, S. Z. Wilson, B. K. Waters, J. M. Quarles, R. S. Cameron, S. E. Greggs, J. M. Zerwas, and R. B. Couch.** 1981. Ribavirin small particle aerosol treatment of influenza. Lancet **ii**:945–949.
8. **Koff, W. C., S. D. Showalter, D. A. Seniff, and B. Hampar.** 1983. Lysis of herpesvirus-infected cells by macrophages activated with free or liposome-encapsulated lymphokine produced by a murine T cell hybridoma. Infect. Immun. **42**:1067–1072.
9. **Levandowski, R. A., C. T. Pachucki, M. Rubenis, and G. G. Jackson.** 1982. Topical enviroxime against rhinovirus infection. Antimicrob. Agents Chemother. **22**:1004–1007.
10. **Lopez-Berestein, G., R. L. Hopfer, R. Mehta, K. Mehta, E. M. Hersh, and R. L. Juliano.** 1984. Liposome-encapsulated amphotericin B for treatment of disseminated candidiasis in neutropenic mice. J. Infect. Dis. **150**:278–283.
11. **Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, V. Fainstein, M. Luna, E. M. Hersh, and R. Juliano.** 1983. Treatment with prophylaxis of disseminated infection due to *Candida albicans* in mice with liposome encapsulated amphotericin. Br. J. Infect. Dis. **147**:939–945.
12. **Lopez-Berestein, G., M. G. Rosenblum, and R. Mehta.** 1984.

Altered tissue distribution of amphotericin B by liposomal encapsulation: comparison of normal mice to mice infected with *Candida albicans*. Cancer Drug Delivery **1**:199–205.

13. **McClung, H. W., V. Knight, B. E. Gilbert, S. Z. Wilson, J. M. Quarles, and G. W. Divine.** 1983. Ribavirin aerosol treatment of influenza B virus infection. J. Am. Med. Assoc. **249**:2671–2674.
14. **McIntosh, K., S. C. Kurachek, L. M. Cairns, J. C. Burns, and B. Goodspeed.** 1984. Treatment of respiratory viral infection in an immunodeficient infant with ribavirin aerosol. Am. J. Dis. Child. **138**:305–308.
15. **Mehta, R., G. Lopez-Berestein, R. Hopfer, K. Mills, and R. L. Juliano.** 1984. Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochim. Biophys. Acta **770**:230–234.
16. **Miller, F. D., A. S. Monto, D. C. Delong, A. Exelby, E. R. Bryan, and S. Srivastava.** 1985. Controlled trial of enviroxime against natural rhinovirus infections in a community. Antimicrob. Agents Chemother. **27**:102–106.
17. **Ninomiya, Y., M. Aoyama, I. Umeda, Y. Suhara, and H. Ishitsuka.** 1985. Comparative studies on the modes of action of the antirhinovirus agents Ro 09-0410, Ro 09-0179, RMI-15,731, 4′,6′-dichloroflavan, and enviroxime. Antimicrob. Agents Chemother. **27**:595–599.
18. **Phalen, R. F.** 1984. Inhalation studies: foundations and techniques, p. 222. CRC Press, Inc., Boca Raton, Fla.
19. **Phillpotts, R. J., D. C. DeLong, J. Wallace, R. W. Jones, S. E. Reed, and D. A. J. Tyrrell.** 1981. The activity of enviroxime against rhinovirus infection in man. Lancet **ii**:1342–1344.
20. **Phillpotts, R. J., J. Wallace, D. A. J. Tyrrell, and V. B. Tagart.** 1983. Therapeutic activity of enviroxime against rhinovirus infection in volunteers. Antimicrob. Agents Chemother. **23**:671–675.
21. **Sokal, R. R., and F. J. Rohlf.** 1969. Biometry, p. 143 and 150. W. H. Freeman & Co., San Francisco.
22. **Taber, L. H., V. Knight, B. E. Gilbert, H. W. McClung, S. Z. Wilson, H. J. Norton, J. M. Thurston, W. H. Gordon, R. L. Atmar, and W. R. Schlaudt.** 1983. Ribavirin aerosol treatment of bronchiolitis associated with respiratory syncytial virus infection in infants. Pediatrics **72**:613–618.
23. **Tadakuma, T., N. Ikewaki, T. Yasuda, M. Tsutsumi, S. Saito, and K. Saito.** 1985. Treatment of experimental salmonellosis in mice with streptomycin entrapped in liposomes. Antimicrob. Agents Chemother. **28**:28–32.
24. **Wilson, S. Z., B. E. Gilbert, J. M. Quarles, V. Knight, H. W. McClung, R. V. Moore, and R. B. Couch.** 1984. Treatment of influenza A(H1N1) virus infection with ribavirin aerosol. Antimicrob. Agents Chemother. **26**:200–203.
25. **Wilson, S. Z., V. Knight, R. Moore, and E. W. Larson.** 1979. Amantadine small particle aerosol: generation and delivery to man. Proc. Soc. Exp. Biol. Med. **161**:350–354.
26. **Wilson, S. Z., V. Knight, P. R. Wyde, S. Drake, and R. B. Couch.** 1980. Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. **17**:642–648.
27. **Wyde, P. R., S. Z. Wilson, C.-S. Sun, and V. Knight.** 1984. Interferon aerosol suppression of vesicular stomatitis virus replication in the lungs of infected mice. Antimicrob. Agents Chemother. **26**:450–454.