



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Evaluation of free or liposome-encapsulated ribavirin for antiviral therapy of experimentally induced feline infectious peritonitis

R. C. WEISS, N. R. COX, M. L. MARTINEZ, *Scott-Ritchey Research Center and Department of Pathobiology, College of Veterinary Medicine, Auburn University, Alabama 36849, USA*

Ribavirin, either free in aqueous solution or incorporated into liposomes, was evaluated in 50 specific-pathogen-free kittens after experimental challenge exposure with feline infectious peritonitis virus (FIPV). Ribavirin was administered daily for 10 to 14 days at 16.5 mg kg⁻¹ bodyweight given per os, intramuscularly or intravenously beginning 18 hours after kittens were challenge-exposed with FIPV. All kittens, including ribavirin-treated and untreated kittens, succumbed to FIP. Clinical signs of disease were more severe in the ribavirin-treated kittens and their mean survival times were shortened. The clinical efficacy of free ribavirin given intravenously at a reduced dosage (5.5 mg kg⁻¹ bodyweight) was compared to that of ribavirin incorporated into lecithin-containing liposomes (5 mg kg⁻¹) intravenously. Drugs were given once daily for three consecutive days of each week for three weeks, beginning 18 hours after virus challenge exposure. There was no significant difference either in survival rate or severity of disease between kittens given free ribavirin, liposomal ribavirin or saline only. Because of its intrinsic toxicity and low therapeutic index against FIPV and its marginal antiviral activities in vivo at maximal doses, ribavirin cannot presently be recommended as primary antiviral chemotherapy against FIP.

FELINE infectious peritonitis (FIP) is a fatal immune-mediated disease of cats caused by a coronavirus, FIPV (Weiss 1989a). FIPV replicates within and disseminates systemically in monocytes and macrophages, particularly those of blood, liver, spleen, lymph nodes, lung, bone mar-

row and mesothelium (Ward 1970, Weiss and Scott 1981a,b, Weiss 1989a,b). Effective immunisation against FIP had not been reported until the recent report by Gerber et al (1990) that a vaccine consisting of an attenuated temperature-sensitive mutant strain of FIPV was efficacious in protecting kittens experimentally challenge exposed with FIPV. However, there is still no effective therapy for FIP; although immunosuppressive drugs have induced some temporary clinical remissions, the disease is considered generally to be untreatable (Pedersen 1978, 1988).

In vitro antiviral studies have indicated that FIPV is susceptible to human or feline interferons (Weiss and Toivio-Kinnucan 1988, Weiss and Oostrom-Ram 1989), ribavirin (Weiss and Oostrom-Ram 1989, Barlough and Scott 1990) and adenine arabinoside and amphotericin B (Barlough and Scott 1990). Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3 carboxamide) is a nucleoside analogue of guanosine with broad spectrum virostatic activities against various RNA and DNA viruses (Nicholson 1984, Reines and Gross 1988). In vitro cytotoxicity studies of ribavirin suggest that there is potential toxicity at antiviral dosages in cats (Weiss and Oostrom-Ram 1989, Weiss 1989b), and in vivo the drug is moderately toxic at dosages of 22 to 44 mg kg⁻¹ bodyweight by various routes in healthy cats (Weiss et al 1993). Experimental trials with interferons in cats inoculated with FIPV have generally been disappointing (Weiss 1989b, Weiss et al 1990).

A novel means of delivering very small and therapeutic dosages of drugs is first to microencapsulate them into liposomes and then inoculate the liposomes intravenously (Alving 1983, MacEwen 1987, Kende et al 1988). Liposomes are

biodegradable unilamellar or multilamellar phospholipid vesicles, similar in structure to plasma membranes, which have a marked affinity after intravenous administration for reticuloendothelial cells, predominantly macrophages of liver, lung and spleen (Alving 1983, MacEwen 1987). Liposomes have been used to deliver chemotherapeutic agents to macrophages in animals infected with viruses, bacteria, fungi or protozoa (Kende et al 1988). In some experimental infections, such as murine leishmaniasis, liposome-encapsulated drugs were about 700 times more effective than non-encapsulated drugs as a form of treatment (Kende et al 1988). Administration of liposome-encapsulated ribavirin in mice infected with Rift Valley fever virus resulted in liver ribavirin concentrations that were fivefold greater than those attained with the same doses of free ribavirin; mice challenge exposed with lethal amounts of virus, moreover, were protected against disease, whereas those treated with the same dose of free drug succumbed to the lethal infection (Kende et al 1985).

Considering the *in vivo* efficacy and *in vitro* toxicity of ribavirin, it was also decided to investigate the use of liposomes as a means of encapsulating and delivering ribavirin. This could have the advantage of lowering the required therapeutic dose and its toxicity, and the drug might be targeted specifically to macrophages in various reticuloendothelial organs, which are the target tissues for FIPV. Previous toxicity studies of ribavirin in the authors' laboratory indicated clinical toxicity occurred at dosages between 11 and 22 mg kg⁻¹ (Weiss et al 1993) and other investigators observed that ribavirin administered intramuscularly at an empirical dosage of approximately 11 mg kg⁻¹ bodyweight was well tolerated for several weeks in cats infected with upper respiratory tract viruses (Dr E. Colby, Dartmouth University Medical School, personal communication, 1990). An initial study was performed and reported here (trial 1) that evaluated the efficacy of free ribavirin against experimentally induced FIP when the drug was used at a dosage of 16.5 mg kg⁻¹ bodyweight. The authors believed this dosage would still be safe when given by various routes or incorporated into liposomes. The objective of the studies reported here was to evaluate ribavirin, administered either alone at a maximal tolerated dose or incorporated into liposomes at a similar dose, as an antiviral treatment for FIP.

Materials and methods

Cats

Fifty healthy 20-week-old specific-pathogen-free kittens of either sex were obtained from a commercial breeding colony (Liberty Laboratories). Kittens were FeLV-antigen-negative (by ELISA) and seronegative for feline coronavirus and feline immunodeficiency virus antibodies. All the kittens were housed individually in federally approved stainless steel cages in temperature, light and humidity-regulated isolation rooms (Scott-Ritchey Animal Isolation Facilities, College of Veterinary Medicine, Auburn University, Alabama); food and water were provided *ad libitum* and litter pans were changed daily. All the animals were used and maintained according to US federal guidelines. Experimental protocols were approved by the Auburn University Institutional Animal Care and Use Committee.

Virus and cell cultures

The DF2 strain of FIPV (ATCC No VR-2004) was used for inoculation of experimental animals and for detection of FIPV-neutralising antibodies. The titre of the FIPV was approximately 10^{7.3} tissue culture infective doses (TCID₅₀ ml⁻¹; by titration, it was determined that the inoculum contained about 10^{5.1} cat lethal doses (LD₅₀) ml⁻¹. Crandell feline kidney (CrFK) cells were propagated in Eagle's minimum essential medium supplemented with 10 per cent heat-inactivated fetal bovine serum, 100 iu of penicillin ml⁻¹, 100 µg of streptomycin ml⁻¹ and 2.5 µg of amphotericin ml⁻¹, along with 2 mM L-glutamine and 1 per cent non-essential amino acids. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of air with 5 per cent carbon dioxide.

Ribavirin

The ribavirin (Lot number 03100785/n-15 of Virazole, supplied by Dr D. Hines, Solvay Animal Health, Mendota Heights, Minnesota) was stored lyophilised at room temperature until diluted before use. Aqueous solutions of ribavirin were made by mixing lyophilised powder with phosphate buffered saline solution (PBSS pH 7.4) and filtering the solution through a 0.2 µm cellulose acetate filter (Corning). Ribavirin solutions were stored at 4°C for a maximum of 72 hours before

use. In vitro screening of aliquots of the ribavirin, using a previously described procedure (Weiss and Ostrom-Ram 1989), confirmed its antiviral activity against FIPV.

Liposomes

A generic lecithin was obtained from a commercial manufacturer (Lot number C-97 of powdered lecithin, supplied by Dr J. Gerber, SmithKline Beecham Animal Health, Lincoln, Nebraska). The lecithin was supplied as a dry, granulated powder which was autoclaved at 15 lb pressure, 121°C for 20 minutes and stored at room temperature before use. Suspensions of empty liposomes or liposome-encapsulated ribavirin were made after reconstituting the dried phospholipid-containing lecithin granules in PBSS or in PBSS containing sterile ribavirin (final concentration of 3 mg ml⁻¹). The solutions were agitated for 10 minutes until the mixture formed a homogeneous creamy-brown consistency. Phase-contrast microscopy of the liposome preparations revealed a mixture of predominantly (about 90 per cent) small unilamellar vesicles 0.2 to 2 µm in diameter along with occasional large multilamellar or onion-shaped vesicles, approximately 5 to 10 µm in size. The calculated lipid:drug ratio of the liposome-encapsulated ribavirin suspension was 1:10 (3 per cent w/v). Liposomes were stored at 4°C for a maximum period of five days during use. Data from the manufacturer indicated that similarly prepared liposomes containing various other compounds were stable for up to several weeks at 4°C (SmithKline Beecham Animal Health Products, unpublished data).

Determination of encapsulation efficiency of ribavirin-containing liposomes

The percentage entrapment of ribavirin by liposomes was determined by lysing the liposomes in detergent, extracting the released ribavirin in the aqueous phase of chloroform/alcohol gradients, and then calculating the concentration of extracted ribavirin spectrophotometrically, using a standard curve. Briefly, liposomes were mixed with ribavirin at a predetermined concentration and then washed in PBSS and ultracentrifuged at 100,000 g to remove unincorporated ribavirin. The pellet was resuspended in PBSS and the liposomal vesicles lysed after adding 5 per cent (v/v) of Triton X-100

(Sigma). Samples of detergent-treated liposomes were visualised under phase-contrast microscopy to confirm that all vesicles were ruptured. The intraliposomal ribavirin was isolated in the aqueous (PBSS) phase of four successive extractions in chloroform/isoamyl alcohol (24:1). The intraliposomal ribavirin concentration was determined spectrophotometrically at an absorbance of 235 nm (maximum ultraviolet absorbance peak for ribavirin in PBSS) by extrapolation from a standard curve of known ribavirin concentrations.

The encapsulation efficiency (EE) of the liposomes for ribavirin, expressed as a percentage of the original drug-loading dose, was 90 per cent. This value was calculated according to the following equation:

$$\begin{aligned} \text{EE} &= \frac{[\text{ribavirin in washed, lysed liposomes}]}{[\text{ribavirin in unwashed intact liposomes}]} \times 100 \\ &= 2.7 \text{ mg ml}^{-1} / 3.0 \text{ mg ml}^{-1} \times 100 \\ &= 0.9 \times 100 = 90 \text{ per cent} \end{aligned}$$

Experimental design of trial

Twenty-four kittens (six kittens per group) were used to evaluate the efficacy of ribavirin given by various routes at 16.5 mg kg⁻¹ bodyweight as an antiviral treatment for FIP.

On the day of inoculation the kittens were sedated, using a combination of telazol and zolazepam hydrochloride (11 mg kg⁻¹ bodyweight, intramuscularly) (AH Robbins) and atropine sulphate (0.05 mg kg⁻¹ bodyweight, subcutaneously). Blood was collected by jugular venepuncture into vacuum-evacuated glass tubes (without anticoagulant) for determination of FIPV neutralising antibodies (VNA) in serum. Kittens were inoculated intraperitoneally with approximately 5 LD₅₀ of FIPV. Eighteen hours after FIPV inoculation at day 0, kittens were given ribavirin per os, intramuscularly or intravenously, or were given a solution of PBSS intravenously. The drug or PBSS was administered to each kitten once daily at 09.00 for the next 13 days (nine days for the intravenous ribavirin and saline-treated groups). All the kittens were examined daily and weighed twice weekly to adjust the drug amount to weight changes. A clinical score, which was correlated inversely with the health of an animal, was calculated daily for each kitten on the basis of a 10-point numerical scale reflecting severity of clinical signs

as follows: anorexia = 5; depression = 5; fever (39.5 to 39.8°C) = 6; ocular or nasal discharges = 6; fever (39.9°C or more) = 7; dehydration = 7; weight loss over baseline (0.25 to 0.4 kg) = 7; hypothermia (37.5°C or less) = 8; pale mucous membranes or slow capillary refill time = 8; vomiting, diarrhoea and/or melaena = 8; weight loss (0.4 kg or more) = 8; laboured breathing = 8; central nervous system signs (seizure, tremors, paralysis, coma) = 9; death = 10. On days 10 and 20 after inoculation, the kittens were sedated and were bled for VNA serum titres as described. Necropsy examinations were performed on all kittens that died during the study and on kittens which were euthanased when moribund with FIP. All surviving kittens were observed clinically for a period of at least nine months after inoculation of FIPV.

Trial 2

In this trial, 26 kittens were evaluated. Twenty-four kittens were inoculated both intranasally and oronasally on day 0 with a total dose of approximately 1 LD₅₀ of FIPV. The kittens (six per group) were treated as follows: ribavirin only (5.5 mg kg⁻¹ bodyweight, intravenously); ribavirin incorporated into liposomes (5.0 mg kg⁻¹ bodyweight, intravenously) along with a small amount (approximately 0.5 mg kg⁻¹ bodyweight, intravenously) or unincorporated or free ribavirin; empty liposomes only (1.8 ml kg⁻¹ bodyweight, intravenously); or PBSS only (virus controls) (1.8 ml kg⁻¹ bodyweight, intravenously). The treatments were given once daily at 09.00 on three consecutive days of each week for three weeks, starting 18 hours after FIPV challenge-exposure on day 0. Two kittens were injected similarly with empty liposomes (1.8 ml kg⁻¹ bodyweight, intravenously) and were not challenge exposed with FIPV (liposome toxicity controls); these kittens were euthanased and necropsied at day 21. Sample collection, clinical scoring and necropsy of kittens in this trial were performed as described for kittens in trial 1.

Virus-neutralising antibody assay

Neutralising antibodies to FIPV-DF2 were measured, using a standard infectivity-inhibition microassay. This assay was performed essentially as described by Weiss et al (1990) except that CrFK cells were used as the indicator cells and viral

cytopathic effects were scored at 72 hours.

Necropsy examination

Kittens were examined for gross changes. Representative sections of all organs and macroscopic lesions were fixed in 10 per cent neutral buffered formalin. The fixed tissues were embedded in paraffin, sectioned at 6 µm, stained with haematoxylin and eosin (H & E), and examined by light microscopy. Touch impressions of bone marrow from each kitten were made on to glass slides and were air-dried and stained, using a modified Wright's stain. Bone marrow smears were examined by light microscopy and evaluated as described previously (Weiss et al 1993).

Statistical tests

Analysis of data was performed using a computerised statistical analysis program (Abstat, Anderson-Bell). Differences in survival rates between groups were evaluated by χ^2 analysis. Mean differences in cumulative survival rates between groups in trial 2 were tested for significance by the Kurskal-Wallis one-way analysis of variance for ranked data, using a non-parametric method. Mean differences in VNA titre, clinical scores, and bodyweights were analysed by the two-tailed Student's *t* test. P values of 0.05 or less were considered significant.

Results

Trial 1: Efficacy of ribavirin by various routes against FIP. Clinical responses

Daily administration of ribavirin (16.5 mg kg⁻¹ bodyweight) per os, intramuscularly or intravenously to kittens for 10 to 14 days after intraperitoneal inoculation of lethal doses of FIPV did not protect kittens against FIP mortality. Mean and median survival times were actually decreased in the ribavirin-treated kittens challenge exposed with FIPV compared to the untreated control kittens (P < 0.05 for kittens given ribavirin orally; Table 1). The predominant clinical signs were fever, depression, anorexia, dehydration, weight loss, icterus, mucous membrane pallor, and hypothermia in the terminal stages of disease. Although fever first occurred on day 1 after inoculation in untreated kittens and kittens given ribavirin intra-

TABLE 1: Survival after intraperitoneal inoculation of FIPV in kittens given ribavirin by various routes (trial 1)

Group	Number alive/ Total	Survival Median (days)	Mean (SD) (days)
Untreated	0/6	21	21.2 (2.2)
Ribavirin* (oral)	0/6	16	16.2 (4.8)‡
Ribavirin* (intramuscular)	0/6	18.5	19.2 (2.7)‡
Ribavirin* (intravenous)	0/5†	19	16.8 (6.1)

*All treatment groups received 16.5 mg of ribavirin kg⁻¹ body-weight once daily for a maximum of 14 days, beginning 18 hours after virus exposure. All kittens were inoculated intraperitoneally with 10^{3.2} TCID₅₀ (about 5 LD₅₀) of FIPV on first day of study

†One kitten died from anaesthetic accident before study

‡Significant decrease compared to untreated kittens, P ≤ 0.01 (two-tailed paired before Student's *t* test, 5 df)

muscularly, fever was delayed by 24 hours or more in three of six kittens given ribavirin orally and in three of five kittens given ribavirin intravenously. Mean clinical scores were increased significantly compared with untreated kittens in all ribavirin treatment groups on day 7 ($P = 0.016$, 0.034 or 0.042 for orally, intravenously or intramuscularly treated kittens, respectively), on day 14 in the oral ($P = 0.023$) and intravenous ($P = 0.021$) groups, and on day 21 in the intramuscular group ($P = 0.013$) (Fig 1). Mean decreases in bodyweight were greater in the infected ribavirin-treated kittens than infected untreated kittens on day 14. The mean weight loss on day 14 in untreated kittens and ribavirin-treated kittens was

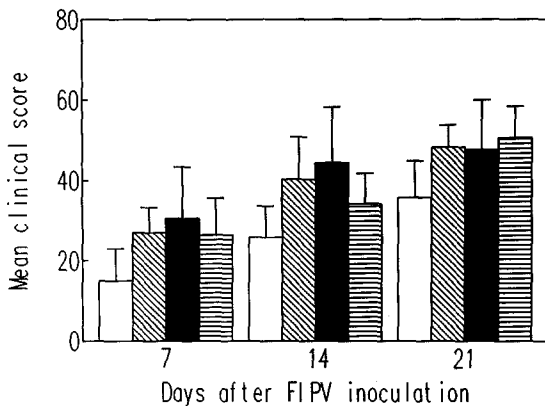


FIG 1: Mean \pm SD clinical scores in untreated kittens (□) and those given 16.5 mg of ribavirin kg⁻¹ bodyweight daily by oral (▨), intramuscular (▤) or intravenous (■) administration, starting 18 hours after intraperitoneal inoculation of 5 LD₅₀ of FIPV (trial 1). Magnitude of clinical score was directly related to severity of clinical signs

0.25 kg (untreated) and 0.66 kg (oral group), 0.40 kg (intramuscular group) and 0.50 kg (intravenous group), respectively ($P < 0.05$ for oral and intravenous groups compared with untreated kittens).

Serological changes

All the kittens had FIPV neutralising antibodies 10 days after virus inoculation (Fig 2). A significant increase in mean VNA titre was observed on day 10 in infected untreated kittens compared with kittens given ribavirin either orally ($P = 0.015$) or intravenously ($P = 0.002$); VNA in kittens given ribavirin intramuscularly tended ($P = 0.062$) to be decreased. Mean \pm SEM VNA titres in untreated kittens and kittens treated orally, intramuscularly, or intravenously were 187 ± 27 and 69 ± 9 , 32 ± 24 and 101 ± 19 , respectively. A rapid increase in VNA titre occurred in all kittens between days 10 and 20. Mean \pm SEM VNA titres at day 20 were 1216 ± 326 in untreated kittens, 1280 in one surviving kitten treated orally with ribavirin, 1173 ± 385 in intravenously treated kittens, and 852 ± 324 in intramuscularly treated kittens. Although the mean VNA titre of intramuscularly treated kittens was less than that of other groups, this difference was not significant. The magnitude of VNA titres did not correlate with positive clinical responses in any group.

Pathological changes

All the kittens had lesions typical of those

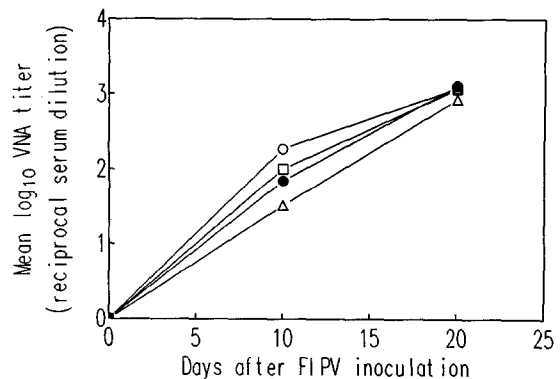


FIG 2: Mean log₁₀ VNA titre in untreated kittens (○) and those treated with 16.5 mg of ribavirin kg⁻¹ bodyweight by oral (●), intramuscular (△), or intravenous (□) administration, starting 18 hours after intraperitoneal inoculation of 5 LD₅₀ of FIPV (trial 1)

induced experimentally after intraperitoneal inoculation of FIPV. In contrast to the untreated kittens, however, kittens treated with ribavirin had a relatively greater incidence of haemorrhagic lesions involving the intestinal tract (small and large bowel). Fifty-five per cent (six of 11) of all the kittens given ribavirin orally or intramuscularly had cerebral haemorrhage. In contrast, none of the infected kittens given ribavirin intravenously and only one infected untreated kitten had haemorrhages in the brain. Kittens in the oral and intramuscular ribavirin groups had a greater number of haemorrhagic lesions than untreated kittens or those given ribavirin intravenously. Kittens in the latter two groups had mild haemorrhages confined mostly to the upper bowel, whereas kittens treated with ribavirin orally or intramuscularly had pronounced haemorrhage or petechiae diffusely in the small and large intestines, liver, heart, lungs, diaphragm, subcutaneous tissues and brain.

Trial 2: Comparison of the efficacy of unencapsulated (free) and liposome-encapsulated ribavirin against FIP. Clinical responses

Significant differences in survival rate after FIPV challenge exposure were not observed between untreated kittens and those treated with either free ribavirin (5.5 mg kg⁻¹, intravenously) or ribavirin

in liposomes (5 mg kg⁻¹, intravenously). The group of kittens treated only with empty liposomes had fewer survivors (none of six) after challenge-exposure with FIPV than untreated kittens (two of six) or those given free ribavirin (three of six) or liposomal ribavirin (two of five); Table 2. These differences, however, were not significant by χ^2 analysis.

The median and mean survival times in kittens receiving free ribavirin were greater than survival times in untreated kittens and kittens given liposomal ribavirin or empty liposomes (Table 2). There was a notable increase in survival rate between four and 13 weeks after virus challenge exposure in the group of kittens given free ribavirin compared to other groups (Fig 3). The survival rates and mean survival times at 13 weeks after FIPV inoculation were 67 per cent (four of six kittens) and 71.2 days for kittens receiving free ribavirin; 33 per cent (two of six kittens) and 50.5 days for untreated kittens; 40 per cent (two of five kittens) 53.4 days for kittens given liposome-encapsulated ribavirin; and 0 per cent (none of six kittens) and 43.2 days for kittens given empty liposomes, respectively.

No significant differences in clinical scores were observed among the groups, although the kittens treated with free ribavirin tended ($P = 0.060$) to have lower scores (that is, fewer clinical signs) on day 30 than untreated kittens (Fig 4). The clinical signs of FIP were similar to

TABLE 2: Survival after oronasal inoculation of FIPV in kittens given liposome-encapsulated or free ribavirin (trial 2)

Group	Number alive/ Total	Survival Median (days)	Mean (SD) (days)‡
Untreated (PBSS)	2/6	36	66.8 (23.3)
Liposomes only	0/6	34	43.2 (9.8)
Ribavirin only	3/6	99	96.3 (21.7)
Ribavirin/ liposomes	2/5†	32	73.0 (27.4)

*Treatment groups received 5.5 mg of ribavirin kg⁻¹ bodyweight intravenously once daily for three consecutive days at the start of each week for three weeks, beginning 18 hours after virus exposure. Liposomes (with or without ribavirin) or PBSS alone were given intravenously at a dosage of 1.8 ml kg⁻¹ bodyweight. All kittens were inoculated oronasally with 10^{2.5} TCID₅₀ (about 1 LD₅₀) of FIPV on first day of study

†One kitten died from cardiac arrest during liposome administration during second week of study and was excluded from data

‡Mean survival time calculated on basis of survival at 20 weeks postinoculation

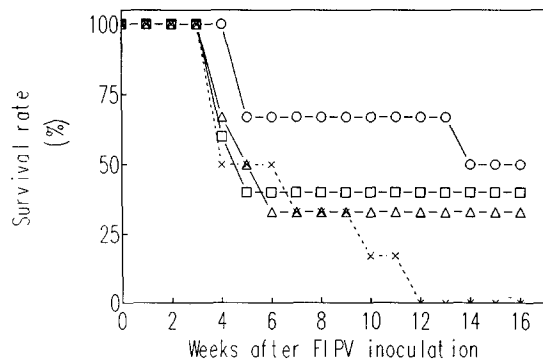


FIG 3: Effect of intravenous administration of free ribavirin, liposomal ribavirin, empty liposomes or PBSS on survival of kittens inoculated with FIPV. Eighteen hours after being inoculated oronasally with about 1 LD₅₀ of FIPV the animals were either (Δ) injected once daily with PBSS (untreated), (○) injected once daily with 5.5 mg of free ribavirin kg⁻¹ bodyweight, (□) injected once daily with 5.0 mg of liposomal ribavirin kg⁻¹ bodyweight, or (X) injected once daily with an equivalent volume (1.8 ml kg⁻¹ bodyweight) of liposome suspension without ribavirin (trial 2)

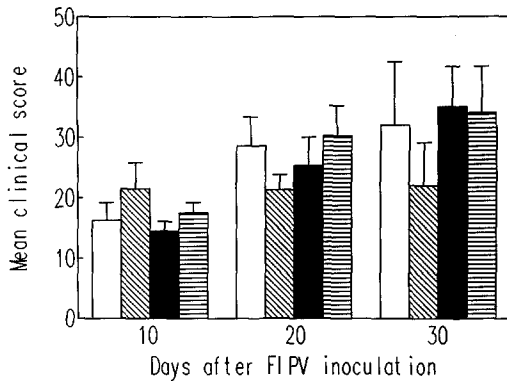


FIG 4: Mean \pm SD clinical scores in untreated kittens (\square) and those given either (\square) 5.5 mg of free ribavirin kg^{-1} bodyweight, (\square) 5.0 mg of liposomal ribavirin kg^{-1} bodyweight, or (\blacksquare) empty liposomes only, starting 18 hours after oronasal inoculation of about 1 LD₅₀ of FIPV (trial 2)

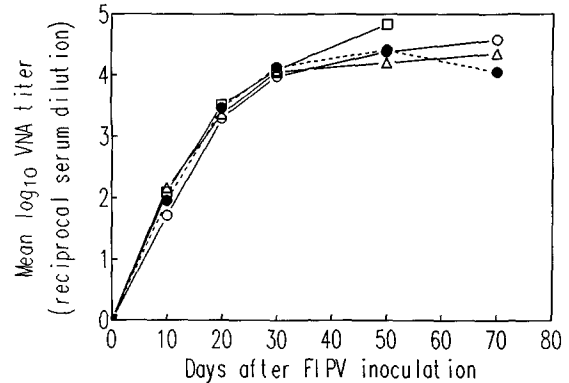


FIG 5: Mean \pm SD VNA titres after oronasal inoculation of about 1 LD₅₀ of FIPV in kittens inoculated intravenously with (\bullet) PBSS only (untreated), (\triangle) 5.5 mg of free ribavirin kg^{-1} bodyweight, (\circ) 5.0 mg of liposomal ribavirin kg^{-1} bodyweight, or (\square) empty liposomes only (trial 2)

those described in the kittens of trial 1. Kittens given liposome-encapsulated ribavirin had a non-significant decrease in mean bodyweight (0.8 kg) on day 30 compared with weight loss in either untreated kittens (0.6 kg), kittens given empty liposomes (0.4 kg) or kittens receiving free ribavirin (0.6 kg). Kittens treated intravenously with liposomes (with or without ribavirin) frequently had acute respiratory distress, including dyspnoea and apnoea, during treatment; these reactions abated when the rate of delivery was considerably decreased. One kitten (A3) that was pre-sedated with tiletamine had acute respiratory failure during liposome administration and died after cardiac arrest on day 14. Necropsy examination of this kitten revealed early FIP lesions in the lungs, liver haemorrhage, and haemoperitoneum. Two uninfected kittens that received equivalent dosages of empty liposomes (1.8 ml kg^{-1} bodyweight, intravenously) remained healthy and did not lose weight or show other signs of disease.

Serological responses

All the kittens seroconverted to FIPV on day 10, and the VNA titres increased progressively during the course of the disease (Fig 5). Although the VNA titres of kittens given free ribavirin tended to be lower, particularly during the first five weeks after challenge exposure than those of other groups, no significant differences in the magnitude of antibody responses between groups were observed.

Pathological changes

All the kittens which died or were killed (17) had lesions typical of FIP. Lesions included vasculitis, perivascularitis, fibrinonecrotising and pyogranulomatous inflammation, and haemorrhage in serosal and parenchymal organs, including the respiratory tract (17 of 17 kittens), lymph nodes (17 of 17), liver (15 of 17), intestinal tract (15 of 17), kidneys (14 of 17), spleen (14 of 17) and central nervous system (10 of 17). Fifteen kittens had haemorrhages in various organs, most commonly affecting the intestinal tract (13 kittens). Eleven kittens had thymic atrophy. Nine of 17 kittens (seven of which died before day 35) showed lesions characteristic of the effusive form of FIP, including thoracic or abdominal effusions (or both). While the extent of the lesions varied markedly among individual kittens, there were no notable differences either in type or severity of lesions (including haemorrhage) among the treatment groups. Kittens given ribavirin only, which on the average survived longer than other kittens, tended to have a slightly higher incidence of chronic inflammatory or non-effusive-type FIP lesions.

Examination of bone marrow impression smears and formalin-fixed marrow sections revealed that FIPV challenge exposed untreated kittens had an increase in bone marrow cellularity, including relative increases in megakaryocytes and osteoclasts; there was a moderate myeloid left shift and normal numbers of erythroid precursors. Kittens given ribavirin only had increased bone

marrow cellularity, a slight myeloid left shift, decreased numbers of erythroid precursors, and either normal or decreased numbers of megakaryocytes. Kittens given liposomal ribavirin had increased bone marrow cellularity with marked megakaryocytosis and a slight myeloid left shift, whereas those given empty liposomes only had increased bone marrow cellularity, erythroid hypoplasia, erythrophagocytosis and either increased or decreased numbers of megakaryocytes. Kittens given empty liposomes only and not challenge exposed with FIPV had no notable changes in bone marrow.

Discussion

The likelihood of drug toxicity at relatively high doses of ribavirin in cats infected with FIPV was predicted on the basis of the low therapeutic index of ribavirin in FIPV-infected feline cell cultures (Weiss and Ostrom-Ram 1989). Based on the findings of previous toxicological studies, it was believed that a dose of 16.5 mg of ribavirin kg⁻¹ bodyweight given alone at various routes in kittens challenge exposed with FIPV would still be sufficiently tolerated and thus allow the authors to evaluate independently its antiviral properties apart from any significant drug toxicity (trial 1).

Kittens treated daily for 10 to 14 days with 16.5 mg of ribavirin kg⁻¹ bodyweight per os, intramuscularly or intravenously after challenge exposure with lethal doses of FIPV (trial 1), however, were not protected against virus-induced disease and mortality. The ribavirin-treated kittens actually had more severe weight loss and other clinical signs of disease, decreased survival times and more severe haemorrhagic lesions after FIPV challenge exposure than untreated kittens, suggesting clinical toxicity at this dosage. Despite this increased severity of clinical signs, ribavirin-treated kittens had some delay in onset of fever after virus inoculation and had decreased mean VNA responses on day 10, suggesting some antiviral activity by ribavirin *in vivo*. The delayed onset of clinical signs and reduced VNA titres in ribavirin-treated kittens after FIPV challenge exposure, however, may have been caused by initial immunosuppressive or myelotoxic effects of the drug rather than direct antiviral actions. A similar effect on early clinical disease and antibody titres was observed in FIPV experimentally infected kittens treated daily with immunosuppressive doses of

interferon- α in a previous study (Weiss et al 1990). Ribavirin toxicity in cats has been demonstrated previously *in vitro* and also *in vivo* at higher dosages than those reported in these trials (Povey 1978 a, b, Weiss and Tovio-Kinnucan 1988, Weiss and Ostrom-Ram 1989, Weiss et al 1993). The toxic effects of ribavirin in kittens, namely thrombocytopenia and suppression of bone marrow erythroid and myeloid precursors (Povey 1978a, Weiss et al 1993), may have increased the severity of clinical disease induced by FIPV in kittens of the present study and aggravated some of the clinicopathological findings of FIP including thrombocytopenia, lymphopenia and anaemia (Weiss and Scott 1980, Weiss 1989a, 1991). Although complete blood and platelet counts were not carried out in the kittens of this study, the findings of more severe and disseminated haemorrhagic lesions in the kittens treated with ribavirin compared to untreated kittens strongly suggested that the pathological effects induced by FIPV, and the toxic effects induced by ribavirin, were additive. Similarly, ribavirin at much higher dosages (25 mg kg⁻¹ bodyweight, given three times daily per os) enhanced the severity of disease, lesions (including severe haemorrhage) and clinicopathological findings in kittens infected with feline calicivirus (Povey 1978a). Because ribavirin alone seemed too toxic to use at dosages likely to be therapeutic, the authors attempted to deliver smaller doses of the drug specifically to tissues and cells infected with virus, using ribavirin-encapsulated liposomes as a carrier-mediated antiviral therapy. The rationale was that effective targeting of liposomes containing ribavirin to target cells of FIPV, namely mononuclear phagocytes in the liver, lung, spleen, bone marrow and lymph nodes (Ward 1970, Weiss and Scott 1981a, b, Weiss 1989a) would result in more precise delivery of antiviral dosages of ribavirin to these cells and thus minimise its distribution to other tissues. It was hoped that the therapeutic dosage would effectively be lowered, thereby reducing toxicity. Previous studies indicated that liposomes are taken up by macrophages *in vitro* and *in vivo* (Bonventre and Gregoriadis 1978, Alving 1983, MacEwen 1987) and that liposomes administered intravenously concentrate in organs with sinusoidal capillaries containing reticuloendothelial cells, such as the liver, spleen, and bone marrow (Ladigina and Vladimirovsky 1986, Popescu et al 1987). Liposome encapsulation of antimicrobial

drugs dramatically improves therapeutic efficacy in various diseases, particularly those associated with disseminated intracellular pathogens (Alving 1983, Kende et al 1985, 1988, Ladigina and Vladimirovsky 1986, Popescu et al 1987) and may reduce bone marrow toxicity of antiviral agents such as 3'-azido-3' deoxythymidine (AZT) (Phillips et al 1991).

In trial 2, both the challenge dose of FIPV and the concentration of ribavirin administered to kittens was lowered. The rationale for lowering the challenge dose of FIPV was to maximise the chances that a positive treatment effect from either free or liposome incorporated ribavirin would be observed and that the kittens would not be over-challenged with virus; it is generally recognised that antivirals often fail at high inocula of challenge virus. The concentration of ribavirin was lowered both to minimise toxicity and also to compare the effect of targeting low doses of drug in liposomes with the effect of equivalent doses of free drug. Unfortunately, the FIPV-infected kittens treated with liposome-encapsulated ribavirin were not protected against induced FIP. Kittens given empty liposomes and challenged with FIPV actually had an increased mortality rate compared to untreated kittens or those given liposomal ribavirin. The reason for the decreased survival in kittens given empty liposomes was unclear; liposome-mediated toxicity or perhaps enhancement of FIPV replication or immunopathological effects secondary to release of cytokines from liposome-activated macrophages may have occurred. Curiously, kittens given equivalent volumes of liposomes with ribavirin had a better survival rate than kittens given empty liposomes only, suggesting that the antiviral effects attributed to ribavirin in these cats may have counterbalanced the deleterious effects of the liposomes. The fact that kittens that had not been challenge exposed with FIPV which were given empty liposomes only did not have notable gross or microscopic changes in their tissues, including bone marrow, argues against a profound toxic effect of the liposomes; haematological or biochemical changes, however, were not quantified in these kittens. Another explanation for the lack of efficacy observed with liposome-encapsulated ribavirin could be that the liposomes were not distributed effectively to the sites of viral replication, or that the amount of ribavirin incorporated into liposomes was suboptimal. The dosage of ribavirin used to load the liposomes (5.5

mg kg⁻¹ bodyweight), of which 90 per cent was incorporated, was not discernibly toxic. This dosage was also not therapeutic, and moderately higher dosages (for example, 16.5 mg kg⁻¹ bodyweight) were apparently too toxic, exacerbating clinical disease. It was still unclear why the free ribavirin was more effective than the liposome-incorporated drug at equivalent concentrations, unless there was liposome-mediated toxicity or modulation of macrophages or other cells that somehow affected viral processing or antiviral immunity. Apparently, the liposomes were unable to target effectively sufficient antiviral doses of ribavirin to critical cell populations and thus failed to prevent or treat disease manifestations of FIP. Species differences in drug tolerance (that is, the therapeutic index of ribavirin for FIPV in cats compared to other mammals) may have contributed to the relative lack of efficacy of free or liposome-encapsulated ribavirin-treated animals in other studies (Kende et al 1985). In practice, the therapeutic window observed with dosages of ribavirin used in this study seemed too narrow to be clinically useful if ribavirin is to be considered as primary antiviral treatment of FIP in cats.

Previous *in vitro* studies of ribavirin indicated that even near toxic levels are not totally inhibitory to FIPV (Weiss and Oostrom-Ram 1989). Considering that FIP is essentially an immune-mediated disease initially induced by rapid virus replication in macrophages rather than the classical type of viral disease associated with viral cytopathicity, it is conceivable that even very small amounts of virus persisting *in vivo* during and after ribavirin treatment were sufficient to elicit immunopathological responses and thus cause progressive disease. The authors' intention in using an antiviral drug like ribavirin as primary treatment for FIP in cats in an acute infection model was to suppress initial viral replication before immunopathological responses were induced. Their intention was not to inhibit direct viral cytopathic effects. If immunopathological responses none the less occurred, it was hoped that the suppression of the circulating viral load using antiviral drugs would largely inhibit the direct stimulus for antibody production and thereby diminish the generation of pathogenic immune complexes. Accordingly, the relative amount of complement-mediated inflammation, necrosis and vasculitis would be reduced, thereby allowing the host to recover from the severe immunologically

mediated inflammatory reactions and catabolic effects that characterise the disease. Apparently, the amount of antiviral activity induced by ribavirin in the cats in this study was ineffective in modulating immunopathological responses therapeutically. Arguably, cats with clinical FIP are presented at a later stage of disease when treatment may be attempted than were the cats in this acute experimental study. Conceivably, there is relatively much less viral replication or viraemia and perhaps different degrees or types of immunopathological injury induced in cats with natural disease compared to kittens with experimentally induced disease. An acute infection model, however, is still relevant to the study of natural FIP. Notwithstanding observations that FIPV can be difficult to isolate in clinical specimens of natural disease, particularly non-effusive cases of FIP (possibly because the virus is so markedly cell-associated, complexed strongly with antibody, or perhaps degraded by proteases and other enzymes released during intense inflammatory reactions), it is possible that at least small amounts of virus continue to replicate and circulate in vivo during clinical disease and induce immunopathological lesions via immune complexes or other mechanisms. Failure of antiviral therapy to ameliorate immunopathological disease (by suppressing viral production) in the experimental disease suggests that this approach alone would probably be ineffective in the natural disease where the immunological problems undoubtedly are more advanced.

These findings indicated that concentrations of ribavirin that are sufficiently tolerated in vivo do not effectively treat cats infected with FIPV or protect them against virus-induced mortality. Incorporation of ribavirin into liposomes to target less toxic concentrations of the drug more specifically to macrophages in various infected tissues was also unsuccessful in preventing fatal FIP. Because ribavirin has been shown to induce synergistic antiviral effects against FIPV when combined with other agents such as human interferon- α in vitro (Weiss and Oostrom-Ram 1989) and because some improvement in clinical responses was induced by low dosages of ribavirin in the cats of this study, additional clinical studies of low doses of ribavirin combined with interferon in FIPV-infected cats may be useful. Considering also that host resistance against FIPV is allegedly related to a strong cellular immune response (Pedersen and Black 1983, Weiss 1989a), effective therapy for

FIP as suggested previously may ultimately depend upon the use of multiple drugs, using a combination of antiviral, immunomodulating and anti-inflammatory agents (Weiss 1989b, Barlough and Scott 1990).

Acknowledgements

This paper is published as Auburn University Publication number 2322, College of Veterinary Medicine, Auburn University, Alabama 36849. The work was supported in part by grants from the American Veterinary Medical Association, the Grace Kemper Fund, Solvay Animal Health, Inc, the Birmingham Feline Fanciers Association, and the Scott-Ritchey Research Center. The authors thank Dr J. Wright for assistance with statistical analysis, Dr M. Boudreaux for evaluating bone marrow cytology and T. Oostrom-Ram, A. Gentry and M. Daniels for technical assistance. Ms Daniels is a third-year veterinary student and recipient of the Birmingham Feline Fancier's summer research fellowship.

References

- ALVING, C. R. (1983) Delivery of liposome-encapsulated drugs to macrophages. *Pharmacology and Therapeutics* **22**, 407-424
- BARLOUGH, J. E. & SCOTT, F. W. (1990) Effectiveness of three antiviral agents against FIP virus. *Veterinary Record* **126**, 556-558
- BONVENTRE, P. F. & GREGORIADIS, G. (1978) Killing of intraphagocytic *Staphylococcus aureus* by dihydrostreptomycin entrapped within liposomes. *Antimicrobial Agents and Chemotherapy* **13**, 1049-1051
- GERBER, J. D., INGERSOLL, J. D., GAST A. M., CHRISTIANSON, K. K., SELZER, N. L., LANDON, R. M., PFEIFFER, N. E., SHARPEE, R. L. & BECKENHAUER, W. H. (1990) Protection against feline infectious peritonitis by intranasal inoculation of a temperature sensitive-FIPV vaccine. *Vaccine* **8**, 536-542
- KENDE, M., ALVING, C. R., RILL, W. L., SWARTZ, G. M. & CANONICO, P. G. (1985) Enhanced efficacy of liposome-encapsulated ribavirin against Rift Valley fever virus infection in mice. *Antimicrobial Agents and Chemotherapy* **27**, 903-907
- KENDE, M., GANGEMI, D. J., LANGE, W., EPPSTEIN, D. A., KREUTER, J. & CANONICO, P. G. (1988) Carrier-mediated antiviral therapy. In *Applied Virology Research*, Vol 1. Ed E. Kurstak, R. Marusyk, F. Murphy. New York, Plenum Publishing, pp 241-264
- LADIGINA, G. A. & VLADIMIRSKY, M. A. (1986) The comparative pharmacokinetics of ^3H -dihydrostreptomycin in solution and liposomal form in normal and *Mycobacterium tuberculosis* infected mice. *Biomedical Pharmacotherapy* **40**, 416-420
- MACEWEN, E. G. (1987) Current immunotherapeutic approaches in small animals. *Proceedings, 10th Annual Kal Kan Symposium* **10**, 47-52
- NICHOLSON, K. G. (1984) Properties of antiviral agents (Part 2). *Lancet* **1**, 562-564
- PEDERSEN, N. C. (1978) Feline infectious peritonitis. *Proceedings of the 45th Annual Meeting of the American Animal Hospital Association* **45**, 125-146
- PEDERSEN, N. C. (1988) Feline Infectious Diseases. Goleta, California, American Veterinary Publications. pp 45-59

- PEDERSEN, N. C. & BLACK, J. W. (1983) Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. *American Journal of Veterinary Research* **44**, 229-234
- PHILLIPS, N. C., SKAMMENE, E. & TSOUKAS, C. (1991) Liposomal encapsulation of 3'-azido-3'-deoxythymidine (AZT) results in decreased bone marrow toxicity and enhanced activity against murine AIDS-induced immunosuppression. *Journal of Acquired Immune Deficiency Syndrome* **4**, 959-966
- POPESCU, M. C., SWENSON, C. E. & GINSBERG, R. S. (1987) Liposome-mediated treatment of viral, bacterial, and protozoal infections. In *Liposomes: From Biophysics to Therapeutics*. Ed M. J. Ostro. New York, Marcel Dekker, pp219-251
- POVEY, R. C. (1978a) Effect of orally administered ribavirin on experimental feline calicivirus infection in cats. *American Journal of Veterinary Research* **39**, 1337-1341
- POVEY, R. C. (1978b) In vitro antiviral efficacy of ribavirin against feline calicivirus, feline viral rhinotracheitis, and canine parainfluenza virus. *American Journal of Veterinary Research* **39**, 175-178
- REINES, E. D. & GROSS, P. A. (1988) Antiviral agents. *Medical Clinics of North America* **72**, 691-715
- WARD, J. M. (1970) Morphogenesis of a virus in cats with experimental feline infectious peritonitis. *Virology* **41**, 191-194
- WEISS, R. C. (1989a) Feline infectious peritonitis and other coronaviruses. In *The Cat: Diseases and Clinical Management*. Vol 1. Ed R. G. Sherding. New York, Churchill Livingstone, pp 333-355
- WEISS, R. C. (1989b) A virologist's approach to treatment of feline infectious peritonitis. In *Feline Infectious Peritonitis: Current Status*. Ed R. C. Hipple. Lawrenceville, New Jersey. Veterinary Learning Systems, pp 14-19
- WEISS, R. C. (1991) The diagnosis and clinical management of feline infectious peritonitis. *Veterinary Medicine* **86**, 308-319
- WEISS, R. C., COX, N. R. & BOUDREAUX, M. K. (1993) Toxicologic effects of ribavirin in cats. *Journal of Veterinary Pharmacology and Therapeutics* (in press)
- WEISS, R. C., COX, N. R. & OOSTROM-RAM, T. (1990) Effect of interferon or *Propionibacterium acnes* on the course of experimentally induced feline infectious peritonitis in specific-pathogen-free and random-source cats. *American Journal of Veterinary Research* **51**, 726-733
- WEISS, R. C. & OOSTROM-RAM, R. (1989) Inhibitory effects of ribavirin alone or combined with human alpha interferon on feline infectious peritonitis virus replication in vitro. *Veterinary Microbiology* **20**, 255-265
- WEISS, R. C. & SCOTT, F. W. (1980) Laboratory diagnosis of feline infectious peritonitis. *Feline Practice* **10**, 16-22
- WEISS, R. C. & SCOTT, F. W. (1981a) Pathogenesis of feline infectious peritonitis: nature and development of viremia. *American Journal of Veterinary Research* **42**, 382-390
- WEISS, R. C. & SCOTT, F. W. (1981b) Pathogenesis of feline infectious peritonitis: pathologic changes and immunofluorescence. *American Journal of Veterinary Research* **42**, 2036-2048
- WEISS, R. C. & TOIVIO-KINNUCAN, M. (1988) Inhibition of feline infectious peritonitis virus replication by recombinant human leukocyte (α) interferon and feline fibroblastic (β) interferon. *American Journal of Veterinary Research* **49**, 1329-1335

Received August 24, 1992
Accepted February 12, 1993