

Hygromycin B Therapy of a Murine Coronaviral Hepatitis

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Hepatitis caused by mouse hepatitis virus (MHV-A59), a murine coronavirus, is accompanied by direct infection and replication of virus within the liver. We demonstrate here that the aminoglycoside hygromycin B is able to eliminate MHV-A59 infection from mouse peritoneal macrophages and cultured liver cells in vitro and is also able to reduce levels of virus replication and necrotic liver foci in vivo.

The proposed selective entry of hygromycin B into cytopathically infected cells (reviewed in reference 5) and the resultant death of these cells by the inhibition of cell protein synthesis suggests that this compound may be useful as an antiviral drug. Along these lines, we have previously presented evidence (9) in support of the antiviral properties of hygromycin B against acute and persistent *in vitro* infections of mouse hepatitis virus (MHV-A59). The studies described in this report attempt to examine the effect of hygromycin B in systems more relevant to natural MHV infection. Evidence is presented from both *in vitro* studies, using mouse peritoneal macrophages and liver cells, and *in vivo* studies which suggest that hygromycin B has a therapeutic effect in limiting MHV replication and in reducing the numbers of hepatic lesions produced during an *in vivo* infection.

***In vitro* studies with peritoneal macrophages and cultured liver cells.** Macrophages and liver cells are important cell targets for MHV. Since the infectability of mouse macrophages often correlates with *in vivo* susceptibility to MHV (1, 17), we examined MHV-A59 infection in cultures of peritoneal macrophages. Two strains of mice were used, BALB/c and A/J, both of which have been shown to be susceptible to MHV-A59 infection (7, 14). Starch-activated peritoneal macrophages (6) were harvested from 4-month-old BALB/c and A/J mice by repeated washing of the peritoneal cavity with minimal essential medium (MEM) containing 20% fetal calf serum (FCS). The total peritoneal cavity wash was centrifuged at $1,000 \times g$ for 1 min, and the pellet was washed with MEM supplemented with 20% FCS. The final pellet was resuspended in MEM (plus 10% FCS), plated out in eight-well microtiter slides, and incubated overnight to allow the macrophages to adhere to the wells. The monolayers were then infected with MHV-A59 (multiplicity of infection, 0.1) and were treated with various concentrations of hygromycin B. Immunofluorescence was carried out on 95% ethanol-5% acetic acid-treated cultures by using a mouse monoclonal antibody directed against the MHV-A59 large envelope glycoprotein (designated S) that was probed with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G. Virus titers in the range of 10^5 to 10^6 were obtained between 12 and 36 h postinfection (p.i.) from MHV-infected peritoneal macrophage cultures obtained from both BALB/c and A/J mice (Fig. 1A and B). The virus-induced cytopathic effect found in both BALB/c

and A/J peritoneal macrophage cultures was extensive, with 100% fusion of the A/J and BALB/c monolayers occurring by 24 h p.i. (Fig. 1D) and 36 h p.i. (Fig. 1C), respectively. This study indicates that peritoneal macrophages from A/J mice are at least as susceptible to MHV-A59 infection as those from BALB/c mice are.

Hygromycin B drastically reduced MHV-A59 replication and virus-induced cytopathology (to undetectable levels) in peritoneal macrophage cultures derived from both BALB/c and A/J mice (Fig. 1). Curing of the virus infection in macrophages by hygromycin B was confirmed by immunofluorescence. At 42 h p.i., the monolayers were treated for examination by immunofluorescence microscopy and were scored for the presence of viral antigen. Expression of virus proteins in peritoneal macrophages from both strains of mice decreased with increasing concentrations of the drug. Immunofluorescence was noticeably reduced at 0.1 mM hygromycin B and was virtually undetectable at drug concentrations of 0.75 to 1 mM.

The sensitivity of MHV infection to hygromycin B was found to be even more marked in cultured mouse liver cells than was the case in peritoneal macrophages. Liver cells were obtained from BALB/c mice by the procedure of Wiltrout et al. (18). Briefly, livers were perfused with medium (MEM supplemented with 10% FCS) via the hepatic portal vein. After removing the gall bladder and any perfused areas, the liver tissue was minced and digested with a mixture of collagenase (0.25 mg/ml), DNase (0.05 mg/ml), and hyaluronidase (0.25 mg/ml) in serum-free medium for 3 h at room temperature. Following filtration through several layers of cotton gauze and centrifugation at $500 \times g$, the pelleted cells were seeded onto collagen-coated slides and cultured in 35-mm-diameter tissue culture dishes. Cells were then inoculated with MHV-A59 (multiplicity of infection, 1) and were incubated in medium in the presence or absence of various concentrations of hygromycin B for 42 h at 37°C. By using immunofluorescence as a guide to MHV antigen expression, levels of viral antigen were noticeably reduced at 12.5 μ M hygromycin B and were not detectable at drug concentrations of 50 to 100 μ M.

Macrophage and liver cell viability, which was measured by trypan blue exclusion, and macrophage phagocytic function, which was assayed by neutral red uptake (11, 12), were not appreciably affected by the concentrations of hygromycin B given above that were shown to be effective in reducing viral antigen expression (Table 1).

***In vivo* studies with MHV-susceptible mice.** A series of studies in mice was carried out to assess the therapeutic efficacy of hygromycin B *in vivo*. Initial toxicity studies of

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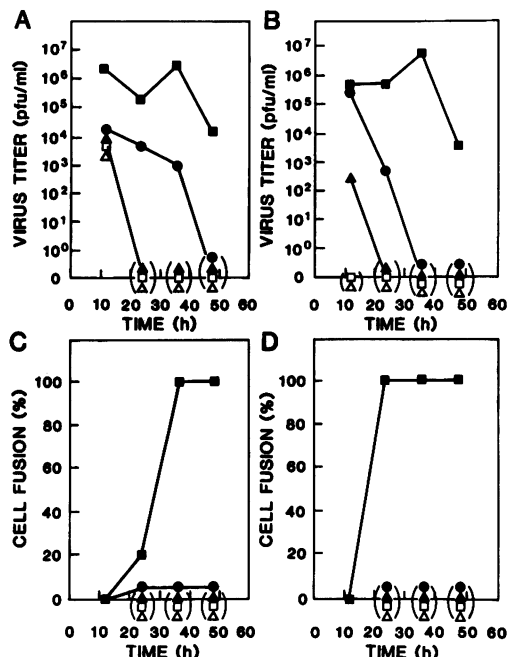


FIG. 1. Outcome of MHV-A59 infection of peritoneal macrophages from two strains of mice that were infected with MHV-A59 and treated with hygromycin B in vitro. Confluent monolayers of peritoneal macrophages from BALB/c and A/J mice were infected with MHV-A59 and treated with hygromycin B at 0 mM (■), 0.1 mM (●), 0.25 mM (▲), 0.5 mM (□), and 1.0 mM (△). Virus titers in the medium were measured every 12 h p.i. in BALB/c (A) and A/J (B) mice, and the cytopathic effect (cell fusion) in BALB/c (C) and A/J (D) mice was also quantitated at these times. Datum points are the averages of determinations on three replicate culture samples. Assay variability generally ranged between $\pm 20\%$ (for virus titers) and $\pm 5\%$ (for cell fusion).

hygromycin B in BALB/c mice revealed an approximate 50% lethal dose of 5 $\mu\text{mol/kg}$ of body weight (2.635 mg/kg). In the following studies, hygromycin B was used at doses of up to 5 $\mu\text{mol/kg}$. Although mice died after receiving that dose, none died after receiving 2.5 $\mu\text{mol/kg}$. Therefore, only the results for surviving animals (i.e., those receiving doses of hygromycin B up to 2.5 $\mu\text{mol/kg}$) were used for analysis. Four-week-old BALB/c mice are susceptible to MHV-A59 infection, following intraperitoneal (i.p.) injection, and rapidly develop necrotic lesions in the liver (14). In our study, BALB/c mice (specific pathogen free and seronegative for MHV-A59 by neutralization test) were given MHV-A59 at 10^6 PFU/20-g mouse by i.p. injection or were mock infected with the equivalent volume of MEM supplemented with 5% FCS. Approximately 30 min later, the mice were given an i.p. injection of hygromycin B at various concentrations in 0.5 ml of 0.9% NaCl. At 3 days p.i., the surviving mice were sacrificed and their livers were processed for histological examination. Paraffin sections from formalin-fixed mouse livers were stained with hematoxylin-eosin. The numbers of lesions were counted and subjected to statistical analysis by using the Statistical Package for the Social Sciences (SPSS, Inc.). Probabilities were calculated by using a one-way analysis of variance test. A probability of <0.05 was assumed to be biologically significant. For virus assays, the same two lobes of each mouse liver were weighed and then homogenized in MEM plus 5% FCS as a 20% (wt/vol)

TABLE 1. Antiviral activity and cytotoxicity of hygromycin B in peritoneal macrophages and liver cell cultures^a

Cell type	Hygromycin B concn (mM)	MHV-specific immunofluorescence ^b	Cytotoxicity (% cells)	
			Trypan blue exclusion ^c	Neutral red uptake ^d
Macrophages	0	++++	100	100
	0.1	+++	93	98
	0.25	++	90	99
	0.5	+	92	92
	0.75	+	89	87
	1.0	—	93	93
Liver cells	0	++++	100	ND
	0.006	++++	100	ND
	0.012	+++	96	ND
	0.025	++	92	ND
	0.05	+	96	ND
	0.100	—	94	ND

^a Results shown are for cells taken from BALB/c mice. Results for A/J mouse cells were similar.

^b MHV-specific immunofluorescence was measured at 42 h p.i. in MHV-inoculated monolayer cultures of macrophages and 24 h p.i. for liver cells.

^c Trypan blue exclusion was measured in uninfected cell monolayers cultured overnight in medium containing the indicated concentrations of hygromycin B. Monolayers were trypsinized, washed, and exposed to trypan blue (0.1% in medium), and cells were examined under a light microscope for enumeration of stained and unstained cells. Results are averages of three determinations, with an approximate sample variability of 5%.

^d Neutral red uptake, as a measure of phagocytosis or pinocytosis, was determined on uninfected cell monolayers cultured overnight in medium containing the indicated concentrations of hygromycin B. Monolayers were exposed to neutral red (0.01% in medium) for 30 min at 37°C, washed, and dye extracted with 1% acetic acid and 50% ethanol. Results are averages of 15 determinations, with an approximate sample variability of 8%. ND, not determined.

homogenate on ice. Viral titers were then assayed by plaque assay by using L-2 mouse fibroblast cells (8).

At 3 days p.i., untreated mouse livers showed some areas of cell fusion, but the major effect was the large numbers of necrotic lesions with sparse polymorphonuclear leukocyte infiltrates. As shown in Fig. 2A, a single injection of hygromycin B reduced the number of lesions in the livers of MHV-A59-inoculated mice. Higher doses of the drug appeared to be slightly more effective at reducing virus-induced lesions. In addition to the single drug dose reported above, we investigated the effects of multiple inoculations (at 12-h intervals) of hygromycin B on the course of MHV-A59 infection in BALB/c mice. Hygromycin B was administered twice daily on days 0, 1, and 2. The multiple-dose regimen resulted in significant reductions in the numbers of liver lesions at all drug concentrations tested (Fig. 2B). Assays for infectious virus recovered from liver homogenates demonstrated a progressive decline in virus titer versus drug concentration administered in the multiple-injection study (Fig. 2C).

To our knowledge, the present report is the first demonstration of an in vivo antiviral effect of hygromycin B against a viral hepatitis. Of particular interest is the fact that hygromycin B is effective against MHV-A59 in vivo at a dose of 2.4×10^{-4} $\mu\text{mol/kg}$, which is much lower than the in vitro concentrations observed to be effective in mouse fibroblasts (9), peritoneal macrophages, or cultured liver cells (this study). This difference may be due to selective accumulation of hygromycin B in certain organs, as has been described with other aminoglycosides (3, 13, 15, 16).

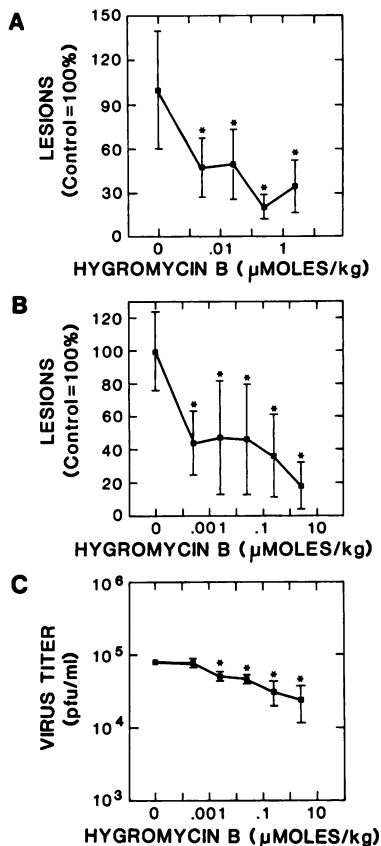


FIG. 2. Effect of single and multiple administrations of hygromycin B on the outcome of infection in MHV-A59-infected BALB/c mice. Four-week-old BALB/c mice were inoculated i.p. with MHV-A59 (10^6 PFU/20-g mouse), and 30 min later they were given a single i.p. injection of hygromycin B at the appropriate concentration (A) or multiple injections every 12 h (B and C). At 3 days p.i., the mice were sacrificed by cervical dislocation, and their livers were removed for histology (A and B) and measurement of virus titer (C). The numbers of lesions in hematoxylin-eosin-stained sections were determined and expressed as a percentage of an untreated MHV-A59-infected control group (four mice in each sample group). All data represent surviving animals (four animals per datum point). No deaths resulted either from MHV infection or from the doses of hygromycin B recorded here. The asterisks denote $P < 0.05$ (one-way analysis of variance); error bars denote standard deviation.

The mechanisms underlying the antiviral effects of hygromycin B remain incompletely understood. Hygromycin B does not inactivate the infectivity of MHV to which it is exposed (9). As a translational inhibiting drug to which normal cells are impermeable, hygromycin B has been suggested to selectively penetrate cells which have been rendered permeable by virus infection (2, 5). Although this view has been challenged (4), we have previously demonstrated that mouse fibroblasts infected with MHV-A59 do show alterations in membrane permeability, as indicated by an increased uptake of radiolabeled sodium ions (10). Thus, virus-induced cell membrane permeability changes may underlie the antiviral activity of hygromycin B against MHV-A59 infection, although other mechanisms are possible.

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