SUCCESSFUL TREATMENT OF ENTEROVIRUS-INFECTED MICE BY 2-(α-HYDROXYBENZYL)-BENZIMIDAZOLE AND GUANIDINE*

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2-(α-Hydroxybenzyl)-benzimidazole (HBB)[1] and guanidine are highly selective and potent inhibitors of picornavirus multiplication in cell culture (1, 2), but little is known about their protective effects in virus-infected animals. Animal experiments with HBB or its chemical derivatives and guanidine so far yielded at most marginal protective effects, in most cases a striking antiviral activity was not observed (3–7). The discrepancy between the results in cell culture systems and animals was explained on the basis of rapid emergence of drug-resistant mutants. In fact, drug-resistant mutants can easily be obtained in cell culture (7–9), and isolation of drug-resistant mutants from virus-infected, treated animals has been reported (4, 7).

In view of the high antiviral potency of HBB and guanidine in cell culture we made another attempt in animals under hopefully optimum conditions. First, newborn mice infected with echo virus type 9 were used. The relationship between virus multiplication and occurrence of paralysis in young mice has been studied extensively (10); an outstanding feature is the development of resistance to the virus with increasing age of the animals. Second, HBB in water-soluble form and as D-isomer, the compound of higher antiviral activity (11), was used. Third, HBB and guanidine were injected in combination: both compounds act synergistically (9, 12) and exhibit only limited cross-resistance vs. mutants resistant to one compound (9).

In this paper, the successful treatment of echo virus type 9 and Coxsackie A 9 virus disease in newborn mice by HBB and guanidine, as well as some decisive parameters of treatment, will be described.

Materials and Methods

Viruses. Echo virus type 9, A. Barty (10), and Coxsackie virus A type 9, Woods (8), have been described before. They were propagated in primary cultures of trypsinized kidney tissue from rhesus or African green monkeys (8).

Cell Cultures. Monkey kidney cells were purchased in suspension from Flow Laboratories (Flow Laboratories Inc., Rockville, Md.) and seeded into tubes or plastic Petri dishes in our

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[1] *Abbreviations used in this paper:* GMK, African green monkey kidney tissue; HBB, 2-(α-hydroxybenzyl)-benzimidazole; MEM, minimum essential medium; PD_{50}, 50% paralyzing doses; $TCID_{50}$, 50% tissue culture infective doses.

laboratory. The growth medium consisted of Eagle's minimum essential medium (MEM) (13) with added fetal calf serum to a concentration of 2–5%. The serum had been inactivated at 56°C for 30 min.

For virus titrations and determination of drug sensitivity of virus sometimes a continuous cell line derived from African green monkey kidney tissue (GMK) was used, kindly supplied by Dr. H. Lennartz, Hamburg, FRG. It was maintained in serial passages by growth in MEM with 10% fetal calf serum.

Infectivity Titrations. Virus titrations in tube cultures were essentially done as described before (8). The maintenance medium for primary cultures was MEM, for GMK cell cultures MEM with 2% fetal calf serum. Concentration of infective virus was expressed in terms of 50% tissue culture infective doses ($TCID_{50}$) per 0.1 ml.

Plaque assays were performed on primary monkey kidney or GMK cell monolayers. After 1-h adsorption at room temperature the inoculum was removed and the plates overlaid with 5 ml of a mixture consisting of equal volumes of 1.8% agar and two times concentrated reinforced Eagle's MEM (14) with 2 or 5% fetal calf serum. Infectivity titers of unadapted echo 9 or Coxsackie A 9 virus in GMK cultures were usually 2–3 times lower than in primary cultures, but all titrations from the same experiment were done simultaneously in the same cell system.

Drug Sensitivity Tests. They were done in tube cultures or by plaque technique as described previously (8, 15).

Neutralization Tests. The technique followed a standard tube neutralization test which has been described in detail previously (16).

Compounds. HBB was used throughout in its D-(−) optical isomeric form (11) as hydrochloride salt. It was kindly synthesized for us by Farbwerke Hoechst AG, Hoechst, FRG. Guanidine, as hydrochloride salt, was purchased from E. Merck, Darmstadt, FRG.

Mice. NMRI mice were either purchased from an animal supply house or reared in the Institute's animal quarters. Mice were infected, less than 24-h old, by subcutaneous inoculation of virus dilutions in 0.02 ml vol. The mice were observed for 21 days at least once a day, and clinical manifestations as well as deaths were recorded. Concentration of virus in mice was expressed in terms of 50% paralyzing doses (PD_{50}) per 0.02 ml.

Standard treatment of mice consisted of subcutaneous inoculations of 0.02 ml vol of saline (0.9% sodium hydrochloride in pyrogen-free water) with or without compounds as indicated, beginning at the time of virus inoculation and extending for usually 10 days. The inoculations were administered into the region of the forearm two times daily, with the right and left side alternating. The standard concentrations of compounds were 10 mM HBB plus 100 mM guanidine for combined treatment, for HBB alone 10 mM HBB, and for guanidine alone 100 mM guanidine.

As indicated in the text, isolation of more mouse-virulent echo virus 9 was achieved by passage in Swiss albino mice. These were kindly provided by ASTA-Werke AG, Brackwede, FRG.

Results

Protective Action of Combined Treatment against Echo Virus Type 9 Disease. Litters of newborn mice were infected with 1–5 PD_{50} of cell culture-grown echo virus 9, corresponding to about 2×10^6 plaque-forming units (PFU) per mouse. Starting at time of virus inoculation, they were simultaneously treated in 0.02 ml doses two times daily for 8–10 days by subcutaneous inoculations of 10 mM HBB plus 100 mM guanidine, by 10 mM HBB alone, or 100 mM guanidine alone, respectively. Assuming an initial body weight of a mouse of about 1 g and uniform distribution of compound, a final concentration of 200 μM HBB and 2 mM guanidine might be achieved in the animal after one inoculation. These are well tolerated and highly virus-inhibitory concentrations of the compounds in cell culture (8).

The results of 11 experiments extending over half a year are summarized in Table I. In the virus control group more than 90% of the animals became paralyzed and 85% of the paralyzed mice died. In some experiments, the mice of

TABLE I
Effect of HBB/Guanidine Treatment on Echo 9 Virus Disease in Newborn Mice

Virus control	10 mM HBB* + 100 mM guanidine	10 mM HBB	100 mM guanidine
94/102‡ (80/94 died)	4/111 (1/4 died)	20/20 (all died)	15/15 (all died)

* 0.02 ml of drugs inoculated subcutaneously two times daily for 8–10 days, beginning at time of virus inoculation.
‡ Number of mice paralyzed over total number of mice.

the control group were kept untreated; in most cases they were inoculated two times daily with saline, but this apparently did not influence the outcome of the experiment. The protective effect of the combined treatment with HBB plus guanidine is obvious: out of 111 mice only 4 became ill, and 3 of these 4 mice had only a slight muscular weakness of short duration. Even the fourth mouse probably would have survived, but, unfortunately, was eaten by its mother. On the other hand, HBB or guanidine treatment alone were ineffective.

That the protective effect of combined treatment is not limited to relatively low doses of virus is demonstrated in Table II. More virulent variants of virus were prepared by repeated virus passages in Swiss albino mice (10). It can be seen that combined treatment with HBB plus guanidine, according to the schedule outlined above, protects completely against more than 10^3 PD_{50}. HBB alone, even in 20 mM concentration, had no demonstrable protective effect. The clinical success of treatment can be correlated with inhibition of virus multiplication as seen in the following experiment.

Inhibition of Virus Multiplication in Treated Mice. Groups of newborn mice were infected with five PD_{50} of echo virus 9 and treated two times daily for $9\frac{1}{2}$ days with either saline, 10 mM HBB plus 100 mM guanidine, 10 mM HBB alone, or 100 mM guanidine alone, respectively. At indicated times after virus inoculation, two mice of each group were collected and frozen at −20°C. Subsequently, of each mouse a 20% suspension in MEM was prepared and the virus content determined in rhesus monkey kidney cell cultures. In Fig. 1 the geometric means of the titrations are presented. Clearly, a significant inhibition of echo virus 9 multiplication was found in the group treated with HBB plus guanidine, though ultimately also in this group the virus titers reached high levels. However, as known from previous studies (10), this delay in virus multiplication is quite sufficient to prevent echo virus 9 disease in mice. On the nature of the "breakthrough" virus, we shall comment below. It should be stressed that not in all experiments a late virus multiplication occurred in the treated group (see, e.g., Fig. 6). It is also obvious from this experiment that treatment with HBB or guanidine alone had no effect on virus multiplication which corresponds to the lack of clinical improvement under these two treatments.

Modifications of the Standard Schedule of Combined Treatment. Since echo virus 9 multiplication does reach its maximum in newborn mice around 3–4 days (10) (see also Figs. 1 and 6), an attempt was made to abbreviate the combined treatment with HBB plus guanidine. Groups of mice, after inoculation of five PD_{50} of echo virus 9, were subjected to standard treatment, which, however, was cut short to only 5 or 3 days, respectively (Table III). Not only 5 days but even 3

TABLE II

Effect of HBB/Guanidine Treatment of Newborn Mice Inoculated with Higher Doses of Echo 9 Virus

Experiment	PD_{50} inoculated	Saline*	10 mM HBB + 100 mM guanidine	20 mM HBB
7-10-74	$10^{3.5}$	8/8‡	0/4	–
1-02-75	$10^{3.2}$	8/8	0/6	–
14-02-75	$10^{2.5}$	6/6	0/7	8/8

* 0.02 ml of saline or drugs inoculated subcutaneously two times daily for $8^1/_2$–$10^1/_2$ days, beginning at time of virus inoculation.

‡ Number of mice paralyzed over total number of mice.

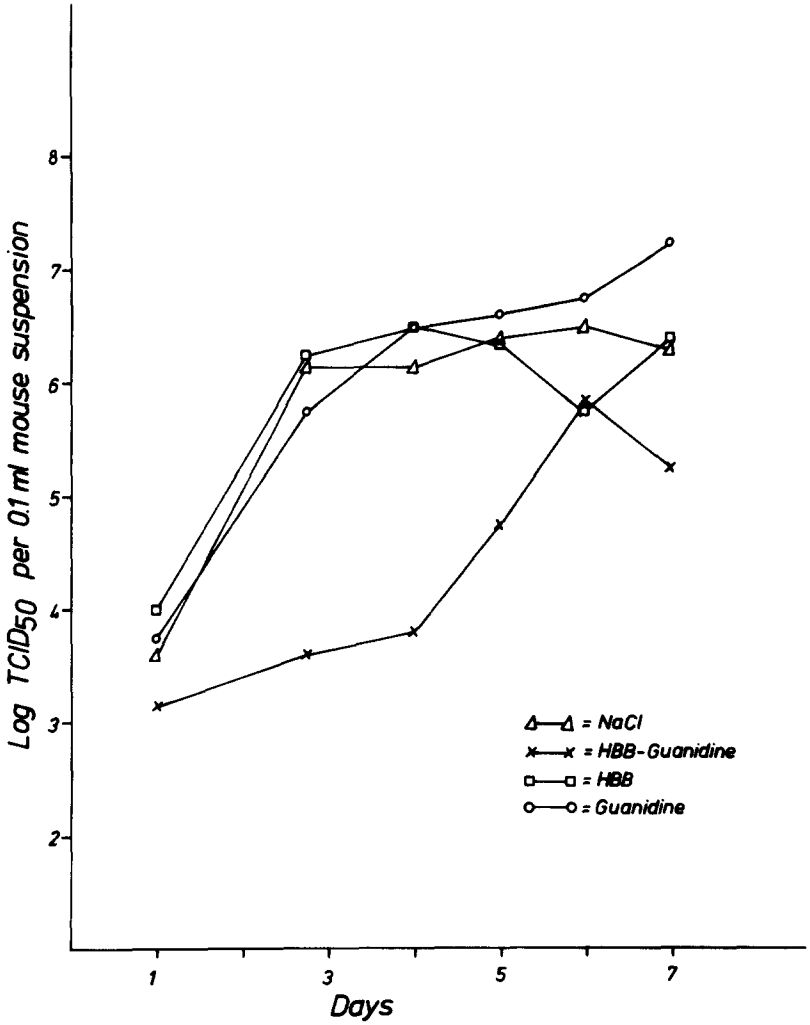

FIG. 1. Multiplication of echo virus 9 in newborn mice, treated two times daily for $9^1/_2$ days with either saline, 10 mM HBB plus 100 mM guanidine, 10 mM HBB alone, or 100 mM guanidine alone, respectively.

TABLE III
Abridged Treatment of Echo 9 Virus-Infected Newborn Mice with HBB/Guanidine

Virus control	10 mM HBB + 100 mM guanidine*	
	For 3 days	For 5 days
5/5‡ (no survivors)	0/3	0/11

* 0.02 ml inoculated subcutaneously two times daily, beginning at time of virus inoculation.
‡ Number of mice paralyzed over total number of mice.

days of treatment beginning with virus inoculation were sufficient for prevention of paralysis. In a complementary experiment of similar design (five PD_{50} per mouse), combined treatment was delayed for 24 or 48 h after virus inoculation, respectively (Table IV). Postponement of treatment for even 48 h was sufficient for protection of the animals.

The dose of drugs administered appeared to be critical (Table V). When only half of the standard dose of combined treatment was inoculated (5 mM HBB plus 50 mM guanidine), no longer complete, though still significant, protection of mice inoculated with five PD_{50} of echo virus 9 was achieved. Quarter doses (2.5 mM HBB plus 25 mM guanidine) were ineffective. Results comparable to those with half of the standard dose were obtained when the standard dose of HBB plus guanidine was administered only once daily instead of twice per day (Table VI).

Toxicity of Treatment. Toxicity experiments were carried out by treating marked mice from single litters with saline or test substances in saline, respectively. Treatment was begun in animals less than 24-h old and maintained for 10–11 days. Inoculations were administered two times daily, 0.02 ml per mouse per injection. Weights of all mice were taken daily. Under these conditions 20 mM HBB or 100 mM guanidine, respectively, never proved toxic. In several experiments 200 mM guanidine led to cyanosis in the mice, and these animals frequently died or were eaten by their mothers.

20 mM HBB plus 200 mM guanidine injected together sometimes also proved toxic, in other experiments it was tolerated and no difference in weight gain to the saline control was detected (Fig. 2). Whether HBB acts somewhat alleviating on guanidine toxicity cannot be decided.

Drug Sensitivity of Virus Recovered from Treated Mice. HBB or guanidine treatment alone of echo virus 9-infected mice is ineffective (Table I), and no inhibition of virus multiplication in the animals thus treated can be demonstrated (Fig. 1). Since drug-resistant mutants can be easily obtained in cell culture, it has been argued that development of drug resistance might be the cause of clinical ineffectiveness. Therefore, echo virus 9 recovered at various times after infection from HBB- or guanidine-treated mice with clinical disease was tested for HBB or guanidine sensitivity and compared with material isolated from corresponding animals of the saline group as well as the virus originally inoculated.

Drug sensitivity was measured as described before (8) by recording the

TABLE IV
Delayed Treatment of Echo 9 Virus-Infected Newborn Mice with HBB/Guanidine

Virus control	10 mM HBB + 100 mM guanidine*		
	At time of virus	24 h post-inoculation	48 h post-inoculation
6/6‡ (no survivors)	0/3	0/9	0/9

* 0.02 ml inoculated subcutaneously two times daily until 10½ days postinoculation of virus.
‡ Number of mice paralyzed over total number of mice.

TABLE V
Effect of Varying Doses of HBB/Guanidine on Echo 9 Virus Disease in Newborn Mice

Saline*	10 mM HBB + 100 mM guanidine	5 mM HBB + 50 mM guanidine	2.5 mM HBB + 25 mM guanidine
8/8‡ (6 survivors)	0/4	2/5 (all survived)	10/10 (no survivors)

* 0.02 ml of saline or drugs inoculated subcutaneously two times daily for 10½ days, beginning at time of virus inoculation.
‡ Number of mice paralyzed over total number of mice.

TABLE VI
Effect of Combined Treatment Given Only One Time Per Day

Saline*	10 mM HBB + 100 mM guanidine	
	Two times daily	One time daily
9/9‡ (no survivors)	0/9	4/9 (7 survivors)

* 0.02 ml of saline or drugs inoculated subcutaneously for 11 days, beginning at time of virus inoculation.
‡ Number of mice paralyzed over total number of mice.

development of viral cytopathic effects in tube cultures under varying concentrations of compound as compared to untreated cultures. In another series of tests reduction of the number and size of plaques with various concentrations of compound in the overlay was measured.

Fig. 3 gives a typical example of HBB sensitivity of echo virus 9 recovered on days 7 to 11 after virus inoculation from mice kept either untreated or treated 2 times daily for 9½ days with 10 mM HBB. No significant difference between virus recovered from mice of either the untreated or HBB group is apparent. Fig. 4 represents corresponding results from guanidine-treated mice. Results of this nature were obtained in large series of experiments using either the tube or plaque reduction method. Similar results were also obtained with HBB- or guanidine-treated mice infected with Coxsackie A 9 virus (see below). Thus, we conclude that development of drug-resistant variants cannot be a significant factor for the failure of treatment when HBB or guanidine is administered

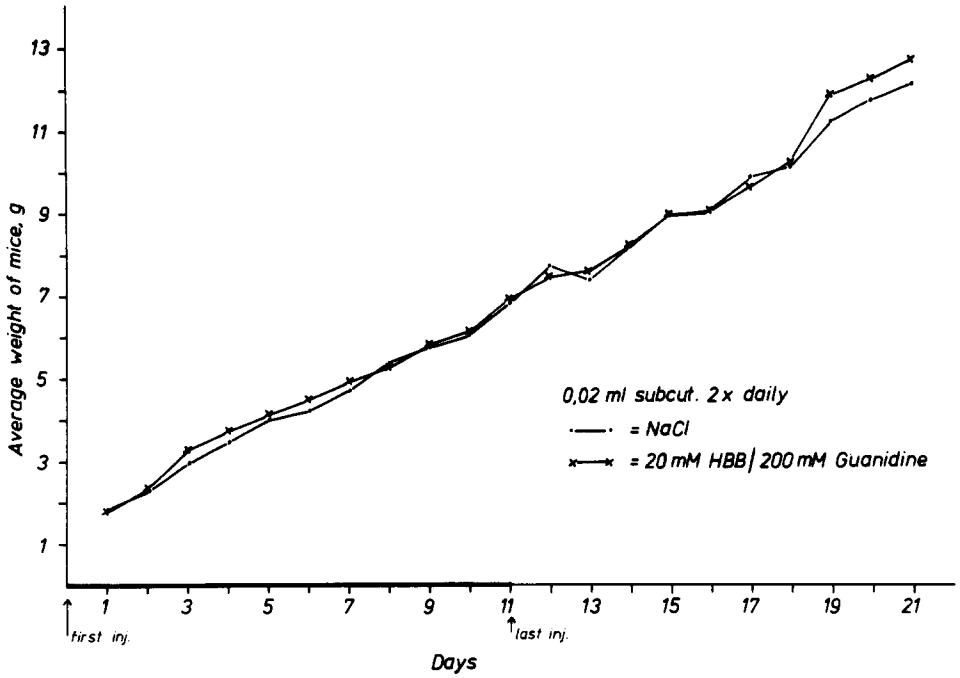

FIG. 2. Weight gain of newborn mice inoculated subcutaneously two times daily for 11 days with 20 mM HBB plus 200 mM guanidine as compared to saline controls.

alone. So far, we recorded only one instance when a sample of Coxsackie A 9 virus from a mouse successfully treated with HBB plus guanidine was significantly more resistant to HBB and guanidine, respectively, than the parent virus. This, however, might be an epiphenomenon, since samples from other mice of the same group exhibited an HBB and guanidine sensitivity like that of the virus inoculum.

Treatment of Mice Infected with Drug-resistant Virus. Though the data presented so far favor the conclusion that the protective effects of treatment are due to the virus-selective activity of the compounds, stronger evidence on this point appeared desirable. We, therefore, tried to prepare an echo virus 9 mutant doubly resistant to HBB and guanidine, respectively. Although it is very easy to isolate drug-resistant mutants in cell culture, these mutants usually are no longer mouse pathogenic. After many futile attempts, we succeeded in obtaining a mutant highly resistant to HBB and guanidine as compared to the parent virus (Fig. 5), exhibiting about one PD_{50} when given undiluted (about $10^{6.7}$ $TCID_{50}$ per 0.1 ml).

With this virus preparation standard growth curves in four litters of mice were performed, two of which were treated 2 times daily with saline, the other two with 10 mM HBB plus 100 mM guanidine. For comparison, drug-sensitive parent virus was used in an experiment of the same design. As can be seen in Fig. 6, multiplication of drug-sensitive virus was strongly inhibited in the drug-treated group and, as expected, residual drug-treated mice not taken for virus titration remained well in contrast to saline-treated mice. On the other hand,

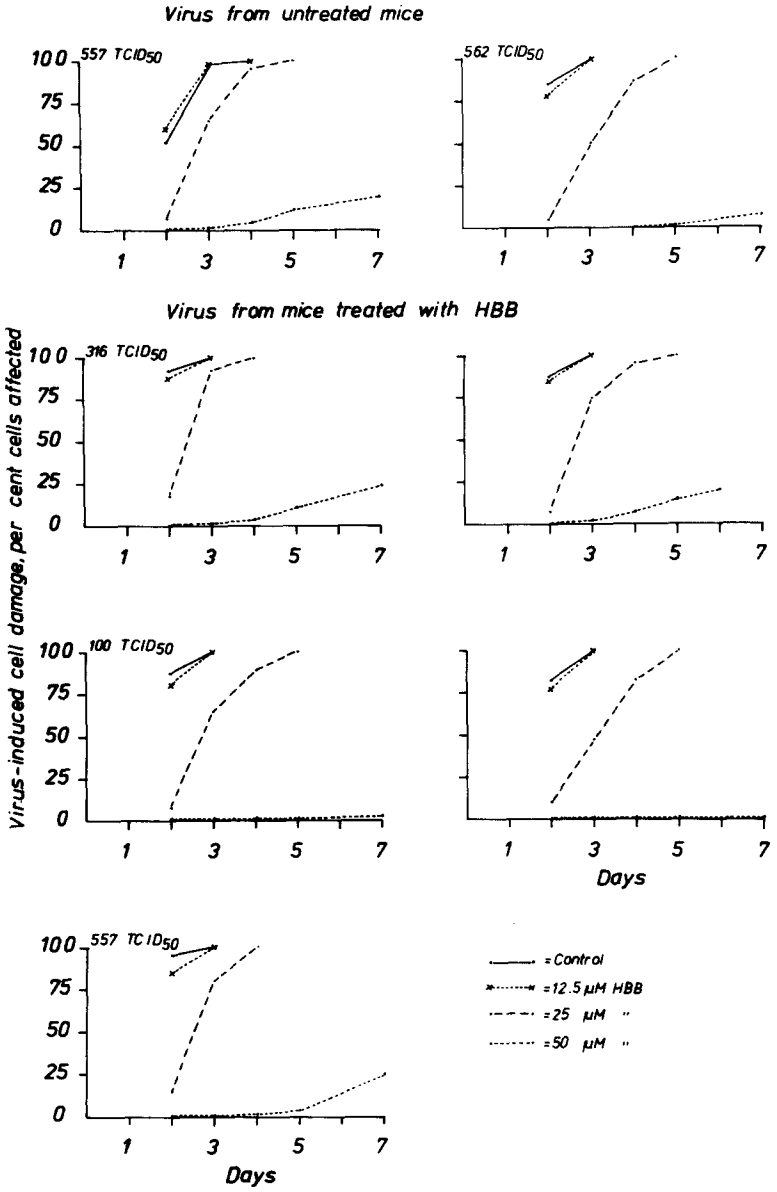

FIG. 3. HBB sensitivity of echo virus 9 recovered from mice kept untreated or treated with HBB.

the multiplication of the HBB-guanidine-resistant echo virus 9 mutant was quite similar in both the saline- and the drug-treated group. Clinically, two out of five mice in the saline group, not taken for virus titration, exhibited paresis; in the drug-treated group two out of four remaining mice were paretic. The results of this experiment leave hardly any doubt that the protection of mice treated with HBB and guanidine is due to the specific virus-inhibitory activity of these compounds.

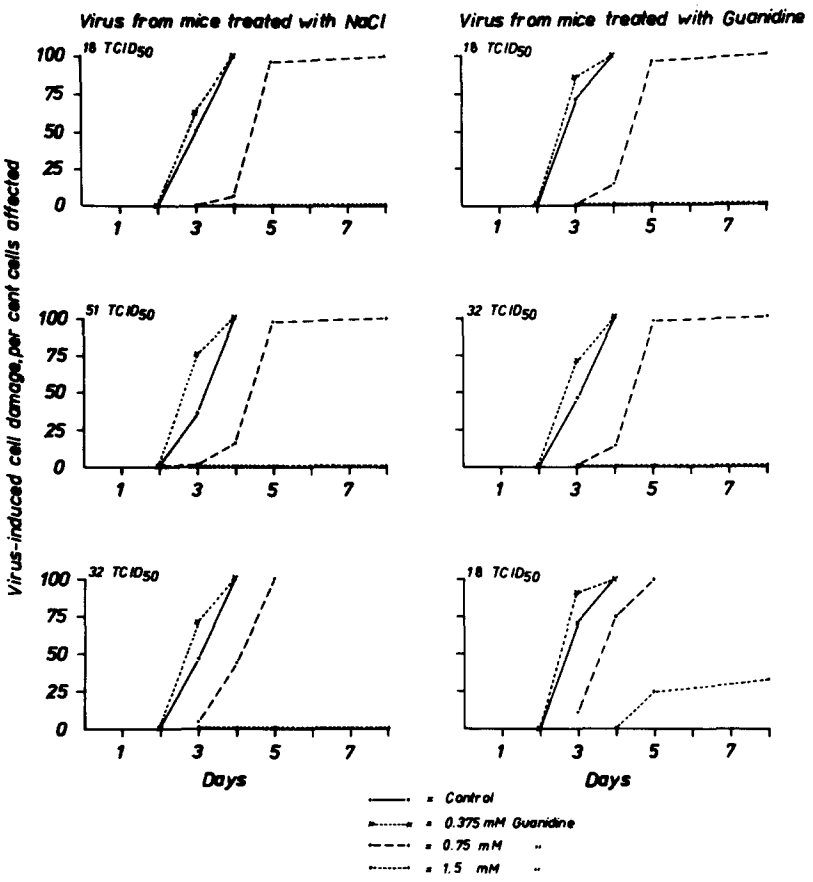

FIG. 4. Guanidine sensitivity of echo virus 9 recovered from mice treated with either saline or guanidine.

Protective Action of Treatment against Coxsackie A 9 Virus Disease. As indicated in Table VII, the protective effect of combined treatment with HBB plus guanidine is not limited to echo virus 9-infected mice, but can also be demonstrated in Coxsackie A 9 virus-infected animals. Furthermore, in the case of Coxsackie A 9 virus infection, treatment with 10 mM HBB alone is clearly effective. On the other hand, guanidine treatment alone is without demonstrable effect. The results on Coxsackie A 9 virus multiplication in treated animals are in line with the clinical findings (Fig. 7): no apparent virus multiplication until at least day 4 after infection in the HBB-guanidine-treated group, and a significant delay in the HBB-treated group as compared to the saline group, a delay quite sufficient for protection of the animals. In guanidine-treated animals, no inhibition of virus multiplication as compared to controls was demonstrated (figure not shown).

The fact that HBB treatment alone protected Coxsackie A 9- but not echo virus 9-infected mice, could be thought of as being due to a higher HBB sensitivity of the Coxsackie A 9 as compared to the echo virus 9, though in previous studies such a difference has not been noted (8, 17). Upon careful re-

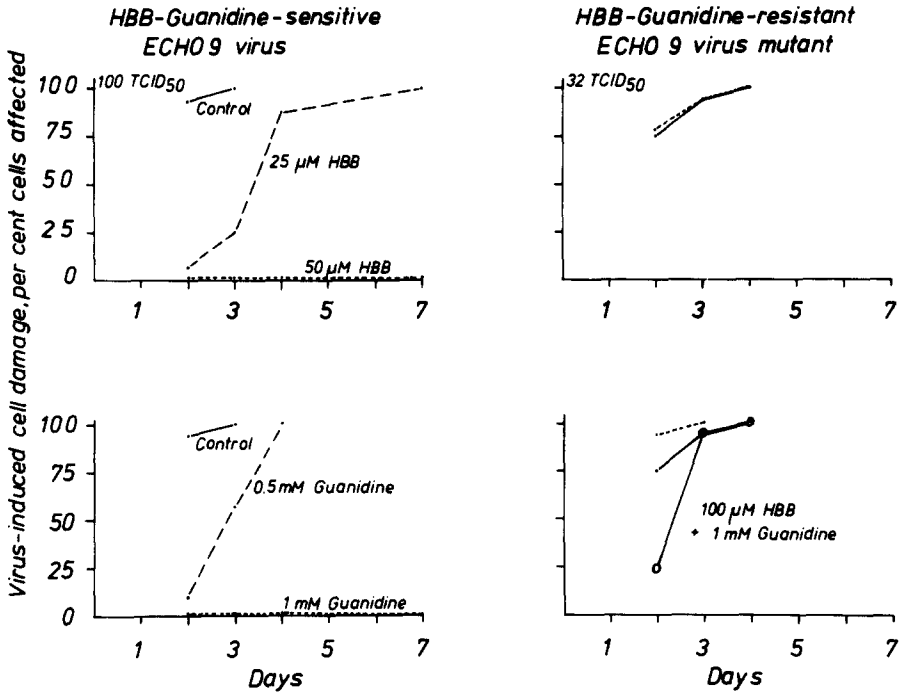

FIG. 5. HBB and guanidine sensitivity of drug-resistant echo virus 9 as compared to the sensitive parent strain. Note in the lower, right field that the mutant is not only completely resistant to 1 mM guanidine, but also highly resistant to the combination 100 μM HBB plus 1 mM guanidine.

examination in simultaneous tests (Fig. 8), no difference in HBB sensitivity between Coxsackie A 9 and echo virus 9 was detected. We conclude that factors other than HBB sensitivity are responsible for the effectiveness of HBB treatment in Coxsackie A 9 virus-infected mice.

Immunity of Echo Virus 9-infected, HBB-Guanidine-treated Mice. Though immunity of previously infected HBB-guanidine-treated mice for obvious reasons cannot be measured directly in challenge studies, two observations strongly suggest that, in fact, a solid immunity does develop. Firstly, sera taken from a number of echo virus 9-infected, HBB-guanidine-treated mice at the age of about 2 mo, all contained echo virus 9 neutralizing antibodies, though, on the average, in somewhat lower titers than those found in untreated or saline-treated animals. Secondly, children born to and fed by mothers which 2 mo previously had been protected by HBB-guanidine treatment from echo virus 9 disease, were solidly immune to homologous virus infection administered within 24 h after birth, but succumbed to Coxsackie A 9 virus challenge.

Discussion

Combined treatment of echo virus 9-infected, newborn mice with HBB plus guanidine protects them from paralysis and death, even when more than 1,000 PD_{50} of virus are inoculated. HBB or guanidine alone are ineffective. Treatment

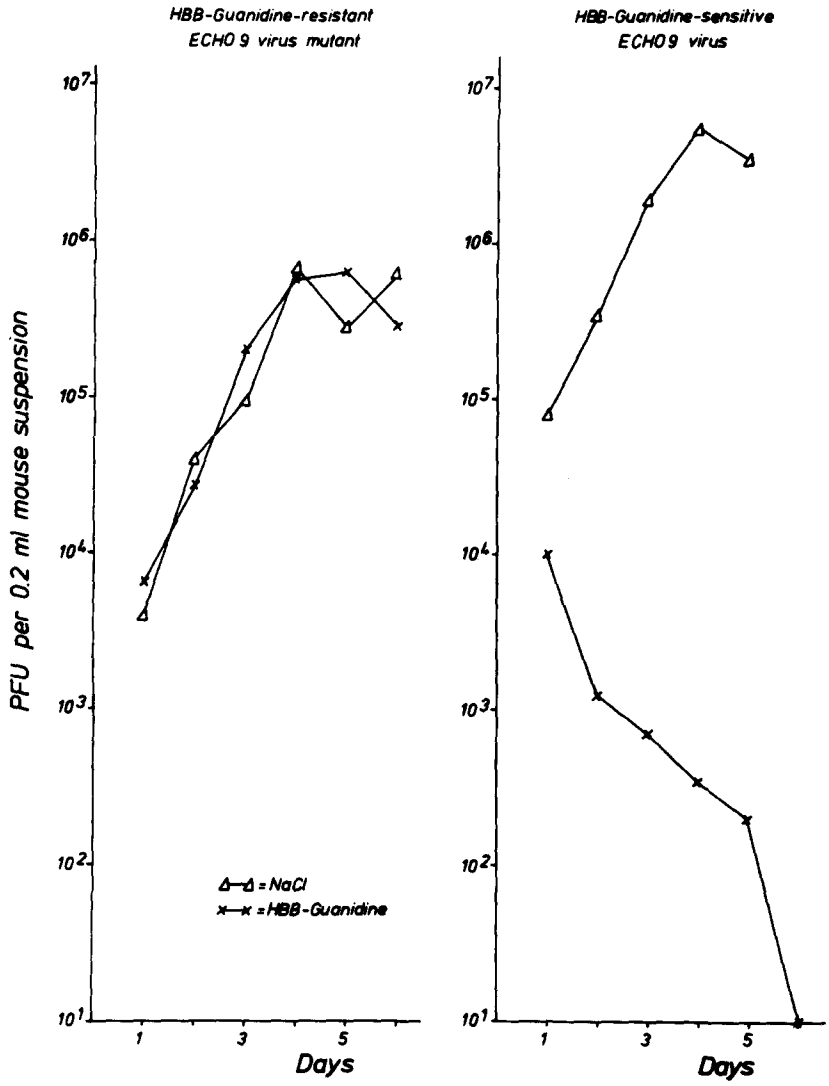

FIG. 6. Multiplication of HBB-guanidine-resistant and drug-sensitive echo virus 9 in mice treated either with saline or HBB-guanidine two times daily for $10^1/_2$ days.

was usually begun at the time of virus inoculation and extended through 10 days, but it may be cut short to 3 days of treatment beginning at time of virus inoculation. Treatment may also be delayed for 48 h after virus inoculation and still be effective.

Coxsackie A 9 virus-infected mice are not only protected from paralysis and death by combined treatment, but also by treatment with HBB alone. Guanidine alone, however, does not protect.

That success of treatment is due to the well-studied antiviral activities of HBB and guanidine is strongly suggested by the following finding. There exists a good correlation between protective activity of the compounds and inhibition of

TABLE VII

Effect of HBB/Guanidine Treatment on Coxsackie A Type 9 Virus Disease in Newborn Mice

Experiment	PD_{50} inoculated	Saline*	10 mM HBB + 100 mM guanidine	10 mM HBB	100 mM guanidine
20-6-74	4.6	9/9‡ (no survivors)	0/9	–	–
5-7-74	4.6	8/8 (5 survivors)	0/5	6/8 (all recovered after slight weakness)	–
6-7-74	4.6	7/7 (no survivors)	–	0/5	3/3 (no survivors)

* 0.02 ml of saline or drugs inoculated subcutaneously two times daily for 10 days, beginning at time of virus inoculation.

‡ Number of mice paralyzed over total number of mice.

virus multiplication in the mice. In particular, HBB-guanidine-resistant mutants prepared in cell culture multiply unaffected in treated mice, and those mice do not respond to treatment.

A most critical factor for success of treatment in echo virus 9-infected mice appears to be a pharmacological one, viz, to reach an adequate concentration of drugs in the target organ, the skeleton muscle. If our standard dose of combined treatment is only halved, either by lowering the concentration of drugs or by reducing the number of injections, still a significant protective effect is achieved, but it is borderline, and quarter doses are ineffective (Tables V and VI). On the other hand, due to toxicity the concentration of guanidine can at most be increased by a factor of two.

Considering the above dose-effect relationships and the very strong synergistic effects of HBB plus guanidine (9, 12), the failure of treatment with either drug alone in the case of echo virus 9 infection appears reasonably explained. Whatever the pharmacological basis, in the broadest sense, may be: at the site of action in the muscle not too effective concentrations of either drug are being reached. More detailed pharmacokinetic studies are under way to shed more light on this point, in particular, whether the conditions in the muscle are comparable to those of cell culture systems.

That the situation may be a more complex one is already suggested by the present study: though in cell culture Coxsackie A 9 virus is not more sensitive to HBB than echo virus 9 (Fig. 8), its multiplication in the muscle is inhibited by administration to the mouse of 10 mM HBB alone, which latter concentration is also clinically effective, whereas in the case of echo virus 9 even 20 mM HBB is ineffective. It may be speculated that in the mouse muscle the sites of attack of Coxsackie A 9 and echo virus 9, respectively, are not the same. This problem is being investigated at present in our laboratory.

Failure of HBB or guanidine treatment alone, at least in our case, appears not

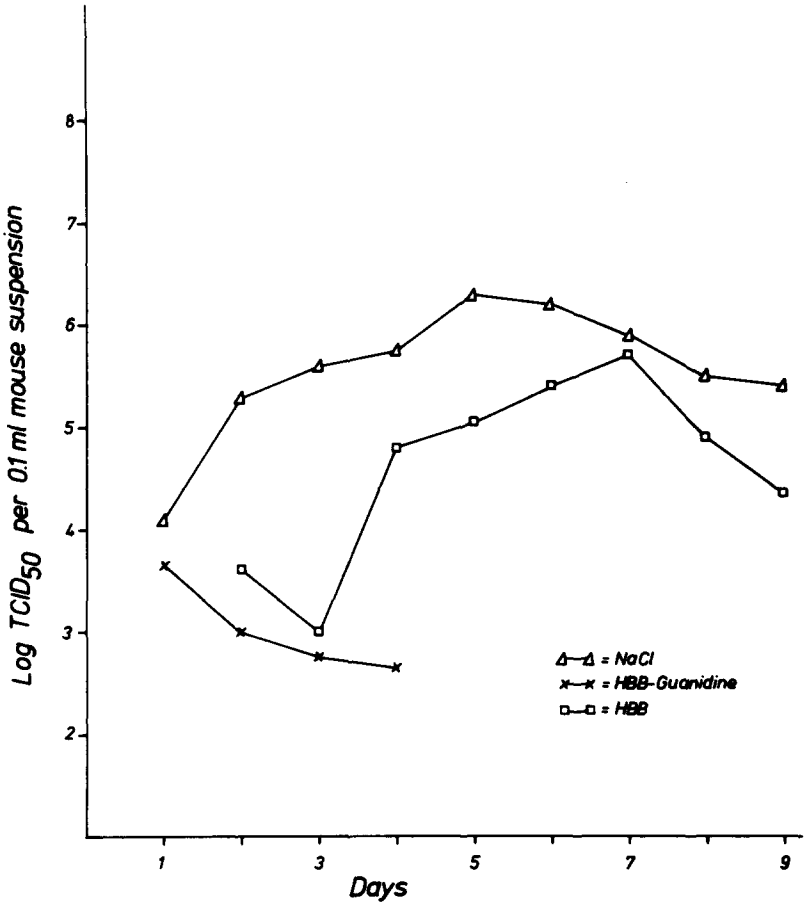

FIG. 7. Multiplication of Coxsackie A 9 virus in newborn mice, treated with either saline, HBB plus guanidine, or HBB alone.

to be due to the rapid emergence of drug-resistant virus mutants as anticipated first by analogy with cell culture studies and by the reports that after treatment of poliovirus-infected monkeys with HBB or guanidine, HBB- or guanidine-resistant mutants of poliovirus were readily isolated (4, 7). In extensive series of experiments we were unable to isolate HBB- or guanidine-resistant mutants from treated animals. Thus, failure of treatment with each compound alone appears not to be a result of development of drug-resistant mutants, and the protective activity of combined treatment in all likelihood is a consequence of the synergism between HBB and guanidine, and not that of the limited cross-resistance between HBB- or guanidine-resistant variants, respectively. O'Sullivan et al. in a study with the 1-propyl derivative of HBB in Coxsackie A 9 virus-infected mice also found no evidence for formation of resistant virus (5). It is not yet clear why guanidine- or HBB-resistant mutants of poliovirus are so readily isolated from guanidine- or HBB-treated monkeys.

Hollinshead and Smith (3) were the first to report therapeutic effects of HBB. They fed the free base of HBB in the diet to mice, infected with poliovirus 2 (MEF_1), and recorded reduction of death (8/11 in the control vs. 1/12 in the

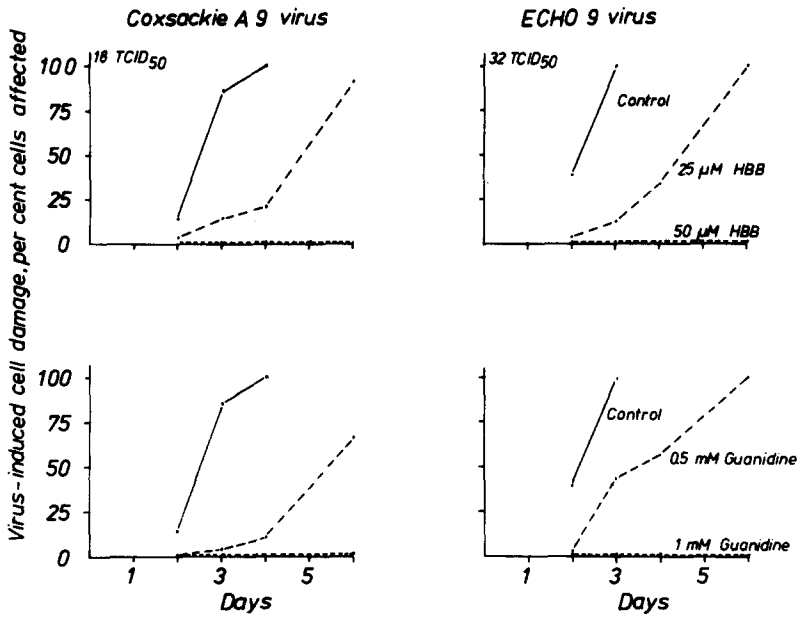

FIG. 8. Comparative drug sensitivity of Coxsackie A 9 virus and echo virus 9.

treated group). It should be emphasized that poliovirus is relatively HBB-insensitive (8), and in another series of experiments HBB did not protect poliovirus-infected mice (4). In the above mentioned study with 1-propyl-HBB in Coxsackie A 9 virus-infected mice (5), the authors gave the free base suspended in corn oil intraperitoneally in doses up to 1.6 mg per mouse per day (our standard dose 0.09 mg per mouse per day). With 3 days of treatment (usually 0.8 mg per mouse per day, distributed in two doses), beginning at time of virus inoculation, they achieved a delay in occurrence of death (with 20 LD_{50} of virus) or a slight protection of 46% survival vs. 25% survival in the control (two LD_{50} of virus). Any possible advantages of the procedures of treatment with the benzimidazole derivatives alone in O'Sullivan's et al. and our study cannot be evaluated properly yet, since the systems may not be comparable. A controlled series of experiments is under way. It should be stated, however, that in cell culture we did not find 1-propyl-HBB superior in its virus selectivity to DL-HBB, the racemic form (Eggers and Tamm, 1963, unpublished results), not to speak of the D-isomer of HBB.

Combined treatment of Coxsackie B virus-infected mice (types 2 and 4) so far gave only marginal beneficial effects (unpublished). In further experiments, the pharmacology of the substances in the organism will be considered in great detail; at present it appears to us a key problem in the pursuit of our studies.

Summary

Echo virus 9- or Coxsackie A 9-infected newborn mice are protected from paralysis and death by combined treatment with nontoxic concentrations of HBB plus guanidine. HBB alone also protects Coxsackie A 9, but not echo virus 9-infected animals, whereas guanidine alone is ineffective in either case. Protection is due to inhibition of virus multiplication via the antiviral activity of these

selective inhibitors. Treatment must be begun at the latest 48 h after virus inoculation. 3 days of treatment are sufficient if started at the time of virus inoculation. Failure of protection after treatment with one compound alone is not due to rapid development of drug-resistant virus mutants. Infected, successfully treated mice may develop a solid immunity.

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References

1. Tamm, I., and H. J. Eggers. 1963. Specific inhibition of replication of animal viruses. *Science (Wash. D. C.)*. 142:24.
2. Eggers, H. J., and I. Tamm. 1966. Antiviral chemotherapy. *Ann. Rev. Pharmacol.* 6:231.
3. Hollinshead, A. C., and P. K. Smith. 1958. Effects of certain purines and related compounds on virus propagation. *J. Pharmacol. Exp. Therap.* 123:54.
4. Fara, G. M., and K. W. Cochran. 1963. Antiviral activity of selected benzimidazoles. *Boll. Ist. Sieroter. Milan.* 42:630.
5. O'Sullivan, D. G., D. Pantic, D. S. Dane, and M. Briggs. 1969. Protective action of benzimidazole derivatives against virus infections in tissue culture and in vivo. *Lancet.* 1:446.
6. Rightsel, W. A., J. R. Dice, R. J. McAlpine, E. A. Timm, I. W. McLean, Jr., G. J. Dixon, and F. M. Schabel, Jr. 1961. Antiviral effect of guanidine. *Science (Wash. D. C.)*. 134:558.
7. Melnick, J. L., D. Crowther, and J. Barrera-Oro. 1961. Rapid development of drug-resistant mutants of poliovirus. *Science (Wash. D. C.)*. 134:557.
8. Eggers, H. J., and I. Tamm. 1961. Spectrum and characteristics of the virus inhibitory action of 2-(α-hydroxybenzyl)-benzimidazole. *J. Exp. Med.* 113:657.
9. Tamm, I., and H. J. Eggers. 1962. Differences in the selective virus inhibitory action of 2-(α-hydroxybenzyl)-benzimidazole and guanidine·HCl. *Virology*. 18:439.
10. Eggers, H. J., and A. B. Sabin. 1959. Factors determining pathogenicity of variants of ECHO 9 virus for newborn mice. *J. Exp. Med.* 110:951.
11. Kadin, S. B., H. J. Eggers, and I. Tamm. 1964. Synthesis and virus-inhibitory activity of D- and L-isomers of 2-(α-hydroxybenzyl)-benzimidazole. *Nature (Lond.)*. 201:639.
12. Eggers, H. J., and I. Tamm. 1963. Synergistic effects of 2-(α-hydroxybenzyl)-benzimidazole and guanidine on picornavirus reproduction. *Nature (Lond.)*. 199:513.
13. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. D. C.)*. 130:432.
14. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. *Virology*. 26:100.
15. Eggers, H. J., and I. Tamm. 1965. Coxsackie A 9 virus: mutation from drug dependence to drug independence. *Science (Wash. D. C.)*. 148:97.
16. Choppin, P. W., and H. J. Eggers. 1962. Heterogeneity of Coxsackie B 4 virus: two kinds of particles which differ in antibody sensitivity, growth rate, and plaque size. *Virology*. 18:470.
17. Eggers, H. J., and I. Tamm. 1961. 2-(α-hydroxybenzyl)-benzimidazole (HBB) as an aid in virus classification. *Virology*. 13:545.