ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1997, p. 687–692 Vol. 41, No. 3
0066-4804/97/$04.00+0

In Vivo Anti-Influenza Virus Activity of a Zinc Finger Peptide

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Received 8 July 1996/Returned for modification 19 September 1996/Accepted 9 December 1996

Matrix protein (M1) is a major structural protein of influenza virus, and it inhibits its own polymerase. A 19-amino-acid peptide, corresponding to a zinc finger region of the M1 sequence of influenza virus strain A/PR/8/34 (H1N1), centered around amino acids 148 to 166, was synthesized. This peptide, designated peptide 6, represents a zinc finger which includes a 7-amino-acid loop or finger and a 4-amino-acid tail at the carboxyl terminus, in addition to the 8 amino acids involved in the coordination of Zn. Three experiments were run to evaluate the activity of peptide 6 on infections induced in mice by influenza A/PR/8/34 and A/Victoria/3/75 (H3N2) viruses. Intranasal (i.n.) treatment of the H1N1 virus infection with 30 or 60 mg/kg of body weight/day, three times daily for 5 days, beginning 4 h pre- or 8 h post-virus exposure, was effective in preventing death, reducing the arterial oxygen decline, and inhibiting lung consolidation. Virus titers in the lungs determined on day 5 were reduced by up to 1.5 $\log_{10}$ in treated groups, but considerable variation in the titers of the recovered virus was seen. The H3N2 virus infection was treated i.n. with 30, 60, or 120 mg of peptide 6/kg/day by using the above-mentioned delayed initiation treatment schedule, and similar protection was seen, although lung virus titers were not reduced in the day-5 assay. Peptide 6 was well tolerated at doses up to 60 mg/kg/day. This zinc finger peptide may provide a new class of antivirals effective against influenza virus.

Influenza A and B viruses cause acute respiratory infection in humans throughout the world. Because currently available vaccines are not completely protective against influenza virus infection, there is interest in the development of antivirals for influenza virus therapy. Presently, the only licensed antiviral drugs for influenza virus are amantadine (1-adamantanamine hydrochloride) and its analog rimantadine hydrochloride (1, 3), and each has limited utility because they are ineffective against type B viruses (7, 10) and are not well tolerated in elderly patients (9). In addition, both of these drugs generate transmissible resistant mutants which further limit their usefulness (11, 17). There is therefore a need for improved therapies for this important disease.

Influenza virus is a negative-strand RNA virus with a segmented genome. The matrix protein (M1), encoded by RNA segment 7, is a major structural component of the virion, occupying the key location between the surface glycoproteins, hemagglutinin and neuraminidase of the envelope, and the ribonucleoprotein complex (2). M1 incorporates into lipid bilayers either as liposomes or as planar bilayer lipid membranes (4) and inhibits its own polymerase, suggesting a possible role of M1 in the regulation of viral replication (22–25). The RNA binding domains of the M1 sequences center around amino acid residues 90 to 109 and 129 to 164 (22). M1 can bind to RNA directly under physiological salt conditions in an in vitro assay for RNA-protein interaction (20); it was proposed that this RNA binding activity may be mediated by amino acids 148 to 162 of the M1 protein, which resemble the zinc binding motif found in a variety of RNA and DNA binding proteins. By viral protein interaction assays, ^{32}P-labeled influenza virus RNA has been shown to be bound to the chemically cleaved M1 peptide corresponding to amino acids 129 to 164 (23), suggesting that this RNA binding activity may be due to the existence of a zinc finger motif. A synthetic peptide based on the M1 sequence centered around amino acids 152 to 166, designated peptide 4, has been shown to inhibit the transcription of influenza virus (14). Peptide 4 showed 38% inhibition of transcription activity at 0.05 μM and contained part of the zinc finger motif. By adding four more residues at the amino terminus, another peptide, peptide 6, was synthesized, and this peptide contained a complete zinc finger and was much more active as a transcription inhibitor than was M1 (14). The 50% inhibitory concentration (IC_{50}) of M1 was 0.7 μM, whereas the IC_{50} of peptide 6 is 0.7 nM (14); thus, peptide 6 is 1,000 times more inhibitory than M1 on a molar basis. Peptide 6 has also been shown to inhibit the cytopathic effect of type A influenza viruses, including H1N1, H2N2, and H3N2 subtypes, and also of type B influenza viruses (15). A low level of activity against vesicular stomatitis virus was also seen (15).

The present paper describes the in vivo activity of peptide 6 against type A (H1N1 and H3N2) influenza viruses in BALB/c mice.

MATERIALS AND METHODS

Animals. Female specific-pathogen-free BALB/c mice weighing 14 to 15 g each were obtained from Simosen Laboratories (Gilroy, Calif.). They were quarantined for 48 h prior to use and maintained on Wayne Lab Blox and tap water. Once the animals were infected, the drinking water was altered to contain 0.006% oxytetracycline (Pfizer, New York, N.Y.) to control possible secondary bacterial infections.

Viruses. Influenza A/PR/8/34 (H1N1) and A/Victoria/3/75 (H3N2) viruses were initially obtained from the American Type Culture Collection (Rockville, Md.). The viruses were passaged 10 times through mice and used as a mouse lung homogenate to enhance the in vivo virulence of each strain. The murine infectivity of each was determined with 14- to 15-g mice exposed intranasally (i.n.) to various dilutions of the virus and then exposed to i.n. treatment with saline following anesthesia to mimic drug delivery three times daily for 5 days. The mice were anesthetized prior to virus and saline exposure with intraperetoneal (i.p.) injections of sodium pentobarbital (60 mg/kg of body weight).

Compounds. The sequence of peptide 6 is from the sequence of M1 protein on the basis of the A/PR/8/34 M1 sequence reported by Winter and Fields (21). Peptide synthesis was performed as follows. The peptide was synthesized by the

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TABLE 1. Effect of i.n. administration of peptide 6 on survival rate and SaO_2 decline of influenza A/PR/8/34 (H1N1) virus-infected mice

Treatment	Dose (mg/kg/day)	Result for treatment group				
		Toxicity control		Infected, treated		
		Surv/total[a]	Host wt change[b] (g)	Surv/total (%)[c]	MTD[d] (day)	(%) SaO_2 (mean ± SD)
Peptide 6 (i.n.)	60	3/3	0.3	8/10 (80)*[e]	4.0	85.3 ± 4.7
Peptide 6 (i.n.)	30	3/3	0.2	5/10 (50)*[e]	4.0	80.6 ± 7.8
Ribavirin (i.p.)	75	3/3	0.6	10/10 (100)**	>10.0**	88.6 ± 2.6
Saline		3/3	−0.2	6/20 (30)	3.0	82.7 ± 8.4
Control		3/3	1.1	3/3 (100)	>10.0	87.5 ± 1.8

[a] Number of surviving mice/number of mice in group.
[b] Difference between initial weight and weight determined 18 h after treatment termination.
[c] * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to saline-treated controls.
[d] Mean time to death.
[e] SaO_2 determined on days 3 and 4.

Merrifield's solid-phase technique (8) on a modified ABI/Perkin-Elmer 431A peptide synthesizer with commercially available N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids attached to modified polystyrene resin and Fmoc-protected amino acids with the following side chain protecting groups: tertiary butyl esters for aspartic acid and glutamic acid, tertiary butyl ether for serine and threonine, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl for arginine, and trityl for histidine, glutamine, and cysteine. After the completion of the synthesis, the peptide was cleaved from the resin with a cocktail containing 88% trifluoroacetic acid, 5% water, 5% phenol (liquid), and 2% diisopropylsilane. Crude peptides were purified with a Waters Delta Prep high-performance liquid chromatography system on a preparative Millipore 25-mm-by-10-cm C_{18} column. A linear gradient extending from 10 to 26% acetonitrile (0.1% trifluoroacetic acid)–water was used. The purity of the peptide was at least 95%.

The peptide was dissolved in sterile physiological saline and used the day the solution was prepared. Each solution was stored in a glass injection bottle at 4°C until used. Ribavirin was obtained from ICN Pharmaceuticals (Costa Mesa, Calif.) for use as a known positive control. This compound was also dissolved in saline for use in this study.

Lung virus determination. Each mouse lung was homogenized to a 10% (wt/vol) suspension in cell culture medium, and various 10-fold dilutions were assayed in triplicate for infectious virus in MDCK cells by using virus-induced cytopathic effect as the end point.

SaO_2 determinations. Arterial oxygen saturation (SaO_2) was determined with the Ohmeda Box 3740 pulse oximeter (Ohmeda, Louisville, Ohio) as previously described (18).

Antiviral experiment design. Three in vivo antiviral experiments were run; two of these studies utilized the A/PR/8/34 (H1N1) virus and therapy which began before or was delayed until after virus exposure. The remaining experiment was run with A/Victoria/3/75 (H3N2) virus and therapy which began prior to virus exposure.

(i) Experiment 1. Mice, anesthetized with sodium pentobarbital, were infected i.n. with $10^{6.0}$ cell culture 50% infectious doses ($CCID_{50}$) of virus/ml. This concentration was shown in a preliminary titration to induce an approximately 80% death rate, moderate lung consolidation, and high virus titers in the lungs. Peptide 6 was administered i.n. to sodium pentobarbital-anesthetized mice in a volume of 0.05 ml three times (8 a.m., 1 p.m., and 6 p.m.) daily for 5 days, beginning 4 h pre- or 8 h post-virus exposure. Ribavirin at a dose of 75 mg/kg/day was injected i.p. to a group of infected mice twice daily for 5 days, beginning 4 h pre-virus exposure. Due to a paucity of drug in this experiment, all infected animals treated with peptide 6, together with the surviving saline-treated controls, were sacrificed on day 5 for assay of lung consolidation and lung virus titer. Five ribavirin-treated animals were also killed at this time, and the remainder were kept a total of 10 days. To determine lung consolidation, lungs were assigned a score ranging from 0 (normal) to 4 (maximal consolidation) and were also weighed. SaO_2 levels were determined on days 3 and 4. Three uninfected mice were treated in parallel with each dose of test compound to serve as toxicity controls; these animals and three normal controls were weighed prior to the start of therapy and again 18 h after the final treatment to determine treatment effects on host weight gain; deaths were recorded daily through 21 days.

(ii) Experiment 2. Due to the high death rate seen in experiment 1, presumably enhanced by the excess fluid introduced into the lungs during treatment, the challenge virus inoculum was reduced to 10^4 $CCID_{50}$ of H1N1 virus/ml. i.n. treatment with peptide 6, used in doses of 120, 60, and 30 mg/kg/day, was three times daily for 5 days, beginning 8 h post-virus exposure. Ribavirin in a dose of 75 mg/kg/day was administered i.p. twice daily for 5 days, beginning 8 h post-virus exposure; in a second group of infected mice, the same dose of ribavirin was given i.n. according to the peptide 6 treatment schedule. In this experiment, 10 mice were kept 21 days, with deaths noted daily and SaO_2 levels measured on days 3 to 10. Five additional mice were killed on day 5 for assay of lung consolidation and lung virus titer. A total of 30 infected mice were treated i.n. with saline in parallel with the peptide 6 treatment group. Twenty of these control animals were observed for death and SaO_2 as in experiment 1, and the remaining mice were killed on day 5 for assay of lung parameters. Toxicity and normal control animals, as described in experiment 1, were included in this study.

(iii) Experiment 3. Mice were infected with $10^{5.8}$ $CCID_{50}$ of H3N2 virus/ml. This virus was pretitrated in mice, treated i.n. with saline according to the Peptide 6 treatment protocol, and the challenge dose was considered a 90% lethal dose. Treatment with peptide 6 utilized the route and schedule of experiment 1, with doses of 120, 60, and 30 mg/kg/day. Ribavirin was administered as in experiment 2, but with i.n. and i.p. therapy begun 4 h pre-virus exposure. The remainder of the study was run as in experiment 2.

Statistical analysis. Increases in survivor numbers were evaluated by the chi-square test with Yates' correction. Mean survival time increases, virus titers, lung weights, and SaO_2 differences were analyzed by the *t* test. Lung consolidation scores were evaluated by ranked-sum analysis.

RESULTS

Tolerance of repeated anesthesia. Repeated use of sodium pentobarbital on the mice, accompanied by the administration of 0.05 ml of saline three times daily for 5 days, showed that such treatment was reasonably well tolerated, causing no deaths of the animals and little host weight loss (data not shown). These data suggested that thrice-daily i.n. peptide 6 treatments given to similarly anesthetized mice would not be stressful to the animals, at least not because of either repeated anesthetic use or additional liquid added to the lungs.

Peptide 6 antiviral study. In the first experiment against the A/PR/8/34 virus, which used treatment begun 4 h pre-virus exposure, the mean time to death of saline-treated animals was 3 days (Table 1), which is a much shorter period than occurred in any of the non-i.n.-treated mice dying of this virus infection. Despite this apparent enhanced lethality, significantly fewer deaths occurred in the animals treated with peptide 6 at higher doses than occurred in the placebo controls. SaO_2 values in the peptide 6- and ribavirin-treated groups were higher than those in the saline-treated controls (Table 1).

The results of the lung parameter assays in this study are seen in Table 2. Consolidation scores in all the treated groups were lower than those in the placebo controls, and mean virus titers in these treated groups were reduced by 1.0 to 1.4 $\log_{10}$. These reductions in virus titer and the reduction in lung scores brought about by peptide 6 therapy at 60 mg/kg/day were statistically significant. Lung weights, usually increased due to pneumonia and the associated fluid in the lungs, were moderately reduced in the peptide 6-treated mice (Table 2).

Ribavirin, administered i.p., induced the positive activity expected, preventing death in all the infected mice (Table 1) and significantly inhibiting lung consolidation, as seen by low-

TABLE 2. Lung parameters of influenza A/PR/8/34 (H1N1) virus-infected mice

Treatment	Dose (mg/kg/day)	Day 5 lung parameter (mean ± SD)		
		Consolidation score[a,b]	Wt (mg)	Virus titer (log_{10}/g)
Peptide 6 (i.n.)	60	1.4 ± 0.7**	218 ± 24	7.0 ± 0.2**
Peptide 6 (i.n.)	30	1.5 ± 1.8**	195 ± 27	7.4 ± 0.2*
Ribavirin (i.p.)	75	0.1 ± 0.2**	116 ± 8.6	5.2 ± 0.3**
Saline		3.6 ± 0.7	245 ± 16.3	8.5 ± 0.1
Control		0.3 ± 0.3	177 ± 5.9	0.0 ± 0.0

[a] A score of 0 (normal) to 4 (maximal consolidation) was assigned to each lung.
[b] * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to saline-treated controls.

ered lung scores and lung weights and reduced virus titers (Table 2).

Peptide 6 was well tolerated at both dosages used in this experiment; as seen in Table 1, all toxicity controls survived the duration of the study and gained weight.

The results for inhibitory effects of peptide 6 when administered 8 h post-virus exposure (experiment 2) are shown in Table 3. Among the saline-treated mice infected with the lesser viral challenge in this experiment, 60% died, with a mean time to death of 8.6 days. None of the infected mice receiving the 120-mg/kg/day dose of peptide 6 died; 20% among those receiving 60 mg/kg/day died, and 10% in the group treated with 30 mg of this compound/kg/day died. Ribavirin, applied i.n., was similarly protective, with 80% of the animals surviving. Those receiving ribavirin i.p. all survived. Among toxicity controls, 1 of 3 mice receiving the high dose of the peptide died on day 2, and a weight loss of 1 g was seen in this group. All the infected mice receiving this dose survived, however. At the lower peptide 6 doses, all toxicity control mice survived but lost 0.7 to 0.8 g in the 5-day treatment period. Ribavirin was reasonably well tolerated when given either i.n. or i.p., with all toxicity control mice surviving but gaining little weight.

SaO_2 values were obtained on days 3 to 10 of this study. In this relatively mild infection, the SaO_2 decline seen was not precipitous but was greatest in the saline-treated infected controls, the mean overall SaO_2 level being 83.9% in these animals. A comparison of the effects of each treatment is seen in Table 3. Both peptide 6 and ribavirin treatment appeared to reduce this SaO_2 decline.

TABLE 4. Lung parameters of influenza A/PR/8/34 (H1N1) virus-infected mice after delayed initiation of i.n. administration of peptide 6[a]

Treatment	Dose (mg/kg/day)	Day 5 lung parameter (mean ± SD)		
		Consolidation score[b]	Wt (mg)	Virus titer (log_{10}/g)[c]
Peptide 6 (i.n.)	120	0.1 ± 0.2	156 ± 8.6	1.2 ± 1.6
Peptide 6 (i.n.)	60	0.1 ± 0.2	138 ± 8.6	2.6 ± 1.9
Peptide 6 (i.n.)	30	0.1 ± 0.2	140 ± 12.9	0.8 ± 0.9*
Ribavirin (i.n.)	75	0.2 ± 0.2	126 ± 21.5	0.0 ± 0.0*
Ribavirin (i.p.)	75	0.2 ± 0.2	126 ± 21.5	0.0 ± 0.0*
Saline		0.6 ± 1.3	128 ± 22.8	1.7 ± 1.9
Control		0.0 ± 0.0	126 ± 5.9	0.0 ± 0.0

[a] Thrice-daily treatment for 5 days, beginning 8 h post-virus exposure.
[b] A score of 0 (normal) to 4 (maximal consolidation) was assigned to each lung.
[c] *, $P < 0.05$, compared to saline-treated controls.

Lung consolidation was not prominent in this experiment at the single time assayed. As summarized in Table 4, neither peptide 6 nor ribavirin appeared to significantly influence either lung score or lung weight. Similarly, recoverable virus titers in the lung were relatively low and in several lungs were below the limits of detection, which resulted in the high standard deviation values shown in Table 4; treatment with the lowest dose of peptide 6 or with i.n.- or i.p.-administered ribavirin significantly reduced these titers (Table 4). The large variance in titers at the two higher dosages of peptide 6 compromise the results, making conclusions regarding effects of peptide 6 at these dosages on virus titers difficult to determine.

The results of the experiment using the A/Victoria/3/75 (H3N2) virus are shown in Tables 5 and 6. Among the infected, saline-treated mice, 95% died, with a mean time to death of 5.9 days (Table 5). Only one (10%) of the infected mice receiving the 120-mg/kg/day dose died, and 30% in the group receiving 30 mg/kg/day died. Ribavirin applied i.n. was somewhat less protective, with 50% of the animals surviving. Those receiving ribarivin i.p. all survived.

The mean SaO_2 values are also shown in Table 5. Figure 1 graphically shows the SaO_2 values over the duration of the time examined and the effects of each treatment on this decline. The placebo control values declined precipitously, with an 8-day mean of 74.2%; in the mice receiving peptide 6, the overall values remained high; the ribavirin i.p. treatment also

TABLE 3. Effect of delayed initiation of i.n. administration of peptide 6[a] on influenza A/PR/8/34 (H1N1) virus-infected mice

Treatment	Dose (mg/kg/day)	Result for treatment group				
		Toxicity control		Infected, treated		
		Surv/total[b]	Host wt change[c] (g)	Surv/total (%)[d]	MTD[e] (day)	% SaO_2[f] (mean ± SD)
Peptide 6 (i.n.)	120	2/3	−1.0	10/10 (100)*	>21.0	87.7 ± 3.3
Peptide 6 (i.n.)	60	3/3	−0.7	8/10 (80)*	2.0	85.1 ± 5.5
Peptide 6 (i.n.)	30	3/3	−0.8	9/10 (90)*	2.0	86.0 ± 5.5
Ribavirin (i.n.)	75	3/3	0.0	8/10 (80)*	2.0	85.7 ± 6.2
Ribavirin (i.p.)	75	3/3	0.2	10/10 (100)**	>21.0	88.1 ± 3.3
Saline		3/3		8/20 (40)	8.6	83.9 ± 4.6
Control		3/3	0.8	3/3 (100)	>21.0	88.3 ± 3.0

[a] Thrice-daily treatment for 5 days, beginning 8 h post-virus exposure.
[b] Number of surviving mice/number of mice in group.
[c] Difference between initial weight and weight determined 18 h after treatment termination.
[d] * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to saline-treated controls.
[e] Mean time to death.
[f] SaO_2 determined on days 3 to 10.

TABLE 5. Effect of i.n. administration of peptide 6[a] on influenza A/Victoria/3/75 (H3N2) virus-infected mice

Treatment	Dose (mg/kg/day)	Result for infected, treated group		
		Surv/total (%)[b,c]	MTD[d] (day)	% SaO_2[e] (mean ± SD)
Peptide 6 (i.n.)	120	9/10 (90)**	9.0	86.7 ± 5.2**
Peptide 6 (i.n.)	60	9/9 (100)**	>21.0**	87.2 ± 2.9**
Peptide 6 (i.n.)	30	7/10 (70)**	6.7	85.4 ± 5.8**
Ribavirin (i.n.)	75	5/10 (50)*	7.0	81.1 ± 5.7**
Ribavirin (i.p.)	75	9/9 (100)**	>21.0**	87.6 ± 3.0**
Saline		1/18 (6)	5.9	74.2 ± 4.7
Control		5/5 (100)	>21.0	87.9 ± 2.4

[a] Thrice-daily treatment for 5 days, beginning 4 h pre-virus exposure.
[b] Number of surviving mice/number of mice in group.
[c] * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to saline-treated controls.
[d] Mean time to death.
[e] SaO_2 determined on days 3 to 10.

kept these values high, but they were lower in the i.n.-administered-ribavirin group. Lung scores were similarly inhibited in the peptide 6- and ribavirin-treated groups, with a mean score of 2.1 in the placebo controls and values of 0 to a maximum of 1.4 in the treated groups (Table 6). This correlated with inhibition of lung weight gain as well, with the placebo-treated animals having a mean lung weight of 257 mg compared to values as low as 188 mg in the peptide 6-treated animals. Lung virus titers are also shown in Table 6. Treatment with the two highest doses of peptide 6 did not significantly reduce these virus titers at the day-5 assay. Ribavirin treatment was somewhat more effective, but the maximum titer reduction seen was only 1.1 $\log_{10}$.

DISCUSSION

Peptide 6 contains a cysteine-histidine-rich sequence, CAT CEQIADSQHRSHRQMV, which is characteristic of the zinc finger motif. This structure may be important in inhibition of viral transcription, perhaps through direct interaction with viral RNA, because the zinc finger structure allows the proteins to bind to DNA and RNA (6). As shown in Fig. 2, the zinc finger domain is centered on a tetrahedral arrangement with a zinc ligand. The central residues 152 through 158 form a potential RNA binding loop or finger. It has been reported that arginine side chains on peptides may commonly be used to recognize specific RNA structures (5). Peptide 6 has two arginines, and these two arginines are highly conserved among influenza A virus M1 proteins. By electrospray ionization mass spectrometry as described by Hutchens et al. (13), it was determined that peptide 6 binds to zinc at the rate of one atom of zinc per molecule of peptide 6 (13a).

The results of the present studies confirm the in vivo antiviral activity of peptide 6 in influenza virus-infected mice. Because of the peptide nature of these products, the experiments were designed to prevent their enzymatic breakdown by avoid-

TABLE 6. Lung parameters of influenza A/Victoria/3/75 (H3N2) virus-infected mice after i.n. administration of peptide 6[a]

Treatment	Dose (mg/kg/day)	Day 5 lung parameter (mean ± SD)		
		Consolidation score[b,c]	Wt (mg)	Virus titer ($\log_{10}$/g)
Peptide 6 (i.n.)	120	0.9 ± 0.4**	188 ± 4.3*	6.3 ± 0.5
Peptide 6 (i.n.)	60	0.8 ± 0.4**	198 ± 17.2*	6.4 ± 0.4
Peptide 6 (i.n.)	30	1.2 ± 0.2*	202 ± 17.2*	6.8 ± 0.4
Ribavirin (i.n.)	75	1.4 ± 0.6	224 ± 43.0	5.3 ± 0.4
Ribavirin (i.p.)	75	0.0 ± 0.0**	146 ± 21.5**	5.8 ± 0.4
Saline		2.1 ± 0.6	257 ± 47.4	6.4 ± 0.3
Control		0.0 ± 0.0**	130 ± 8.6**	0.0 ± 0.0

[a] Thrice daily treatment for 5 days, beginning 4 h pre-virus exposure.
[b] A score of 0 (normal) to 4 (maximal consolidation) was assigned to each lung.
[c] * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to saline-treated controls.

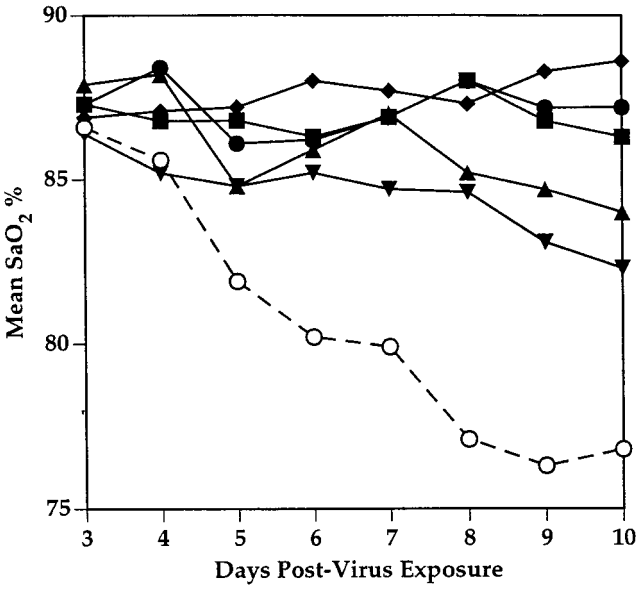

FIG. 1. Effect of i.n. treatment with peptide 6 and ribavirin on SaO_2 in influenza A/Victoria/3/75 (H3N2) virus-infected mice. ■, peptide 6 (120 mg/kg/day); ●, peptide 6 (60 mg/kg/day); ▲, peptide 6 (30 mg/kg/day); ▼, ribavirin (i.n.) (75 mg/kg/day); ◆, ribavirin (i.p.) (75 mg/kg/day); ○, saline. Beginning with day 6, the SaO_2 values for all treated groups were higher than those for the saline-treated control group by a statistically significant ($P < 0.05$ to $P < 0.001$) degree. Standard deviations did not exceed 3% and were generally in the range of 1.5 to 2.5%.

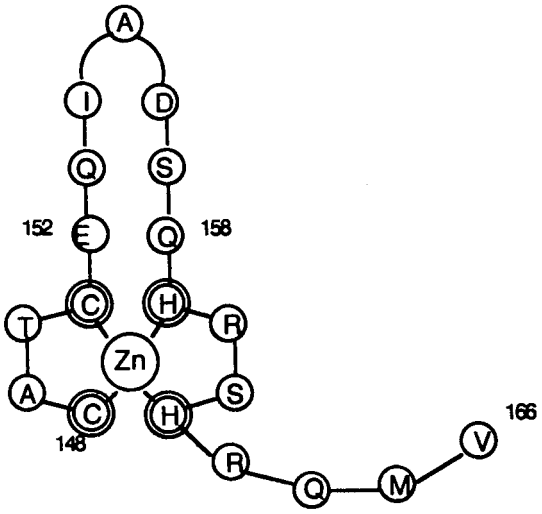

FIG. 2. Schematic folding for a linear arrangement of peptide 6, the zinc finger domain centered on a tetrahedral arrangement of zinc ligand. The central residues 152 through 158 form a potential RNA binding loop or finger.

ing parenteral administration. This was accomplished by using the i.n. route of administration. Preliminary experiments were also run to determine if mice can tolerate repeated anesthesia and i.n. exposure to liquid, and a preliminary virus titration was run to ascertain the most appropriate virus dosage for use in the study. Treatment with peptide 6 appeared to be inhibitory to both the H1N1 and H3N2 influenza A viruses. Further work is needed to determine effects against influenza B virus infections.

It is recognized that the viral challenge used in this study was rather low, being lethal to 60 to 70% of the placebo-treated mice. Such a challenge would tend to enhance the antiviral activity of peptide 6 but more closely resembles the milder infections commonly seen in humans.

An unexpected result was seen in this study in that the saline-treated infected mice died very quickly. This accelerated lethality and death rate could be attributed to the virus spreading through the lung at a higher rate due to excess liquid added to the lungs, which would also contribute to pneumonia developing in the lungs. When the A/Victoria/3/75 virus in mice treated three times daily i.n. with saline was titrated, it was found that approximately 100-fold less virus was needed to cause death in the animals (data not shown). Peptide 6 seemed to give protection against this condition, because in the peptide 6-treated animals these deaths were significantly fewer than those in the placebo controls. Peptide 6 and i.n.- or i.p.-administered ribavirin, when therapy was delayed until 8 h post-virus exposure, were still significantly inhibitory to the disease, indicating therapeutic potential for each. In this delayed-treatment experiment, peptide 6 had better inhibitory activity than ribavirin administered i.n.

The lesser effect of peptide 6 and ribavirin on lung consolidation and virus titers may have been a reflection of the time selected (day 5) in the progression of the infection to assay for these parameters. Ideally, had there been sufficient test compound the effects at various days during the disease would have been monitored. It has been our experience that virus titer reductions often are greatest early in the infection (e.g., days 1 or 2), whereas lung consolidation may reach maximal values later (days 6 to 8), although if a major virus reduction did occur early in the infections, one would anticipate this continuing as long as therapy continued. The choice of day 5 in the present studies was an attempt to compromise on these times to show antiviral effects on all lung parameters and represented the last day of the therapy.

Because the virus titers were not markedly reduced in this study, and indeed not too dose responsive in some cases, one may speculate that the protection of the mice from death due to peptide 6 treatment may be due to some mechanism other than inhibition of viral replication. The in vitro antiviral data described by Nasser et al. (15) indicate, however, a direct inhibition of the infected cell. It is not uncommon to have a significant prevention of influenza virus-induced death in mice without a major inhibition of lung virus titer. We have previously observed this phenomenon, using polyoxometalates (12), and Ryan et al. (16) reported similar findings of virus titer reductions in mice with the neuraminidase inhibitor GG167 to range from 0.8 to 1.1 $\log_{10}$ when assayed on day 5. In the latter study, the virus titers on day 4 ranged from 0.7 $\log_{10}$ below the control to 0.4 $\log_{10}$ above the control, depending on the dosage of GG167 used. Yet in both the polyoxometalate study and the GG167 study, a significant prevention of influenza virus-associated death occurred despite these marginal lung virus titer reductions.

These data indicate that peptide 6 was well tolerated up to a dose of 60 mg/kg/day when given i.n. Lack of toxicity seen at disease-inhibitory doses suggests that peptide 6 may have an acceptable therapeutic index. Further studies need to be run to determine the lowest dose of the compound that will still significantly inhibit influenza virus infection. Such data would further establish the therapeutic window for this material. When given i.n., peptide 6 had a better activity than ribavirin, which was run in parallel at doses approaching the maximum tolerated in mice (19). On a molar basis, peptide 6 (molecular weight, 2,200) is several-fold more active than ribavirin (molecular weight, 224).

There is a need for important new therapies for influenzal disease; as reviewed earlier, amantadine and rimantadine, the only drugs at present available for treatment of this disease, have significant problems limiting their use. The present data indicate peptide 6 to be a first compound in the development of a new class of antivirals for use against this major public health problem.

ACKNOWLEDGMENT

This work was supported by SBIR grant NIH AI134712 to A.K.J. from the National Institute of Allergy and Infectious Diseases.

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