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Chemotherapy and Vaccination: a Possible Strategy for the Control of Highly Virulent Influenza Virus

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The influenza A virus [A/Chicken/Pennsylvania/1370/83 (H5N2)] that caused up to 80% mortality among chickens provided a model system for testing the efficacy of chemotherapeutic agents against highly virulent influenza virus. Amantadine and rimantadine administered in drinking water were efficacious both prophylactically and therapeutically. However, under conditions simulating natural transmission of virus, amantadine- and rimantadine-resistant viruses arose and were transmitted to other birds in contact with the infected chickens, causing mortality. Simultaneous administration of inactivated H5N2 vaccine and amantadine provided protection. Thus, chemotherapy may be useful in the treatment of a highly pathogenic influenza virus outbreak in humans or other animals when used in combination with vaccine.

The highly virulent influenza virus that appeared in chickens in Pennsylvania in 1983 illustrates the potential threat of influenza to animals as well as to humans. The virus was devastating to chickens, causing up to 80% mortality among chickens on some farms and high mortality among turkeys and guinea fowl. The outbreak of disease was caused by an influenza A virus of the H5 subtype [A/Chicken/Pennsylvania/1370/83 (H5N2)] (Chick/Penn). Viruses of this hemagglutinin subtype include A/Tern/South Africa/61 (H5N3) (2) and A/Chicken/Scotland/59 (H5N1) (15), which have also caused high mortality among birds. To eradicate this disease outbreak, quarantine measures were instituted and a state and federal task force was formed to depopulate farms of infected birds. The virus has apparently been eradicated from domestic poultry as a result of the depopulation, in which over 17 million birds were slaughtered at a cost of approximately $61 million.

Amantadine hydrochloride (1-adamantanamine hydrochloride) and rimantadine hydrochloride (methyl-1-adamantanemethylamine hydrochloride) are effective chemotherapeutic agents in the prophylaxis of influenza A infections in humans (4, 8, 13, 19, 20). Efficacy rates of 85% for rimantadine and 91% for amantadine have been reported. Amantadine has also been shown to be effective against influenza A virus infection of horses (3), quail (5), and turkeys (10). In turkeys treated with daily doses of amantadine, infection was prevented or remained subclinical in 80% of birds infected with a highly virulent influenza A virus (10). The appearance of a highly pathogenic virus in chickens in Pennsylvania in October 1983 provided an opportunity for evaluation of these chemotherapeutic agents.

The studies indicate that amantadine and rimantadine are efficacious but that resistant variants of the virus arise, are transmitted to other chickens in contact with the infected birds, and cause mortality. Vaccine plus chemotherapeutic agents prevented the appearance of resistant mutants.

MATERIALS AND METHODS

Viruses and vaccine. The virulent Chick/Penn virus isolated in October 1983, which caused up to 80% mortality among birds in the field and up to 100% mortality after nasal inoculation (10^4 50% egg infective doses [EID_{50}]) into White Leghorn chickens, was used as the virulent virus in these studies. These viruses were provided by James Pearson, Ames, Iowa. A/Mallard/New York/189/82 (H5N2) (Mal/NY) was isolated from an apparently healthy wild mallard duck and was avirulent in chickens. Viruses were grown in 11-day-old embryonated chicken eggs and purified by differential sedimentation through a 25 to 70% sucrose gradient in a Beckman SW28 rotor. The Mal/NY virus was inactivated with β-propiolactone and standardized by single radial diffusion (21).

Infection and treatment of animals. White Leghorn chickens (5 to 6 weeks old) and adult laying hens (>6 months old) were used in these studies. The birds were housed in our P3 containment facility in air-filtered compartments.

Groups of chickens were infected by dropping 0.1 ml of virus containing 10^4 or 10^5 EID_{50} of Chick/Penn virus into the nasal cleft of each chicken; in titrations in adult laying hens this is equivalent to 10^3 and 10^4 chicken infective doses, respectively. Rectal swabs were collected once per day for 3 days after infection, and virus isolation was done in 10-day-old chicken embryos (6). Rimantadine or amantadine was dissolved in water and provided ad lib beginning at the time the birds were infected. Susceptibility to reinfection was determined by administration of 10^4 EID_{50} of virus into the nasal cleft at 14 days after infection and 1 day after stopping administration of a chemotherapeutic agent in the drinking water.

Serological tests. Hemagglutinin (HA) titrations and hemagglutination inhibition (HI) tests were performed on microtiter plates with sera treated with receptor-destroying enzyme. The antigen used in the test was disrupted with Tween 80-ether before use in the test (11).

RESULTS

Chemotherapeutic agents and their excretion. Since commercial chicken farms frequently contain more than 50,000 birds, a method for mass application of the chemotherapeutic agent was investigated. Both amantadine and rimantadine are stable and water soluble (7), and so they were adminis-

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TABLE 1. Prophylactic value of amantadine and rimantadine in chickens infected with Chick/Penn influenza virus[a]

Age of chickens	Chemoprophylactic agent (dose)[b]	EID_{50}	Total no. of birds	No. of birds with virus	No. of birds dead (day of death)	No. of birds with antibody response/total no.	No. of birds susceptible to reinfection/total no.	Mean wt of birds (g)[c]
5 to 8 weeks	None	10^4	10	10	10 (5–6)			
	R (0.1%)	10^4	4	0	1[d] (5)	0/3	NT[e]	247
	R (0.01%)	10^4	10	0	0	1/10	5/6	309
	R (0.002%)	10^4	6	6	1 (9)	5/5	0/5	NT
	A (0.1%)	10^4	4	0	1[d] (8)	0/3	NT	149
	A (0.01%)	10^4	10	0	0	0/10	6/6	310
	A (0.002%)	10^4	6	6	1 (9)	5/5	0/5	NT
Adult (laying hens)	None	10^5	6	6	6 (3–4)			
	A (0.01%)	10^5	6	3	2 (4–5)	4/4	NT	
	None	10^4	8	8	8 (4–6)			
	A (0.01%)	10^4	8	0	0	0/8	NT	

[a] Groups of White Leghorn chickens were infected and treated as described in the text.
[b] R, Rimantadine; A, amantadine.
[c] The mean weight of a group of birds not treated with a chemotherapeutic agent or infected and fed the same food and water was 273 g.
[d] Death due to dehydration and trauma; these birds were not debeaked.
[e] NT, Not tested.

tered in the drinking water. Amantadine (0.01%) supplied in drinking water was rapidly absorbed by the body; by 24 h, significant levels were present in serum (354 ng/ml), muscle (688 ng/g), and liver (2,560 ng/g). The levels increased slightly by day 2 and were maintained; after removal of the drug, the levels in serum and tissue fell almost to zero within 24 h but the level in the white and yolk (2,450 ng/g) of eggs was maintained for at least 3 days.

Prophylactic evaluation. To investigate the prophylactic value of these drugs, young birds (5 to 8 weeks old) and adult laying hens were inoculated with virus, and the chemotherapeutic agent was administered simultaneously. In young birds, neither agent was well accepted at the 0.1% level (Table 1), water intake decreased, and the birds showed a reduction in weight gain as compared with the controls. Despite the poor acceptance of the agents, none of the birds showed clinical signs of infection, and virus was not isolated. At the 0.01% level, both rimantadine and amantadine were well accepted, and the treated birds weighed at least as much as the control group (Table 1). No clinical symptoms were observed in the young birds treated with amantadine or rimantadine, and virus was not recovered. The drugs prevented infection, as indicated by the fact that only 1 of 10 birds treated with 0.01% rimantadine seroconverted. When the chemotherapeutic agent was used at 0.002%, all of the birds were infected: one bird in each group died, and the rest were listless from days 5 to 10 postinfection but recovered; these birds produced high levels of antibody (HI titers of >640). The untreated birds all died by day 6 postinfection with hemorrhage of the legs and a fulminating virus infection with signs of central nervous system involvement. All of the birds treated with 0.01% amantadine were susceptible to reinfection when the drug treatment was discontinued.

In adult birds, 0.01% amantadine was well accepted, and the birds continued to lay eggs. Adult birds treated prophylactically with 0.01% amantadine and infected with 10^4 EID_{50} of virus gave the same results as younger birds, indicating high levels of efficacy. However, at higher doses of virus (10^5 EID_{50}), half of the birds were infected and died, indicating that amantadine does not protect completely against high doses of virus.

Therapeutic evaluation. Studies were done to determine whether amantadine was efficacious if administered subsequent to virus inoculation (Table 2). Amantadine was effective in young birds (5 to 6 weeks); the majority of birds (7/8) infected 24 or 48 h before administration of the drug survived, and half of the birds (2/4) infected 72 h before treatment survived. Most of the birds were listless from days 4 to 8 postinoculation and shed virus in their feces for up to 10 days but recovered and developed high levels of antibodies (HI titer of >1,000). Adult birds infected with Chick/Penn virus before administration of amantadine gave similar results. The level of virus shed in the feces of untreated adult birds was as high as $10^{7.3}$ EID_{50}/g, and similar levels of virus were shed by amantadine-treated birds.

Transmission. The above experiments demonstrate that amantadine is effective in the treatment of chickens experimentally infected with the virulent Chick/Penn influenza

TABLE 2. Therapeutic value of amantadine in chickens infected with Chick/Penn influenza virus[a]

Age of chickens	EID_{50}	Time of infection before amantadine administration (h)	No. of birds	No. of birds dead (day of death)	No. of birds with antibody[b] response/no. surviving
5 to 8 weeks	10^4	24	4	1 (8)	3/4
		48	4	0	4/4
		72	4	2 (6–8)	2/2
		NT	4	4 (5–6)	
Adult (laying hens)	10^4	24	10	0	10/10
		48	10	2 (4)	8/8
		72	10	4 (3–8)[c]	6/6
		NT	4	4 (3–4)	

[a] Groups of White Leghorn chickens were infected with Chick/Penn virus at 1, 2, or 3 days before administration of 0.01% amantadine in their drinking water, as described in the text.
[b] HI antibody titers ranged from 1,000 to 2,000.
[c] Of the 10 birds, 1 died before amantadine was given in the drinking water.

TABLE 3. Efficacy of amantadine and vaccine in the prevention of transmission of Chick/Penn influenza virus in chickens[a]

Treatment of chickens			No. of birds affected/total no. of:				HI antibody titers in surviving contacts[e]
			Infected birds		Contacts		
Virus[b]	Amantadine[c]	Vaccine dose (μg of HA per dose)[d]	Sick	Dead	Sick	Dead	
+	−		2/2 (3–5)[f]	2/2 (4–6)	10/10 (4–6)	10/10 (4–6)	
+	+		0/10	0/10	25/50 (7–18)	18/50 (9–20)	1,200
+	−	18	2/2 (4–5)	2/2 (5)	8/10 (4–6)	7/10 (5–9)	1,000
+	+	18	0/2	0/2	0/10	0/10	200
+	−	9	2/2 (4)	2/2 (4–5)	9/10 (4–6)	8/10 (5–9)	1,500
+	+	9	1/2 (7)	1/2 (7)	0/10	0/10	300
+	−	3	2/2 (3–5)	2/2 (5–6)	10/10 (4–6)	9/10 (3–7)	1,000
+	+	3	0/2	0/2	6/10 (6–15)	5/10 (7–15)	2,000
−	+	18			0/10	0/10	200
−	−	18			0/10	0/10	300

[a] Two birds were infected with virus and 48 h later were put into cages containing 10 adult laying chickens that had received vaccine or amantadine treatment, or both, immediately before exposure. Amantadine treatment was continued for the entire course of the experiment.
[b] Adult White Leghorn laying chickens were inoculated in the nasal cleft with 10^4 EID_{50} of virulent Chick/Penn influenza virus.
[c] Amantadine (0.01%) was provided ad lib in drinking water.
[d] Vaccine was prepared from Mal/NY influenza virus as described in the text.
[e] HI titers give the geometric mean values at 14 days after initial contact with infected birds.
[f] Numbers in parentheses give the day(s) on which birds first showed disease signs or died.

virus but did not prevent virus shedding. The experiments do not mimic natural exposure, and the question remained whether the virus shed in the presence of amantadine would be transmissible to other drug-treated birds. To emulate field conditions, adult laying hens were inoculated with virus, and 48 h later two infected birds were moved to cages containing 10 susceptible birds; all birds were then given amantadine (0.01%) in their water (Table 3). None of the infected birds developed signs of disease, but half of the contacts (25/50) developed severe signs of disease at 9 to 20 days later, and the majority (15/25) of these birds died after developing the severe clinical signs described above. Similar results were obtained when rimantadine was used in transmission studies (results not shown). All of the surviving birds developed high levels of antibodies, indicating that all of the contacts had been infected. The virus recovered from these birds was resistant to amantadine in the plaque assay described by Appleyard (1), and 0.01% amantadine did not prevent infection of chickens with 10^4 EID_{50} of this virus (results not shown). Previous studies have shown that amantadine-resistant strains occur at a relatively high frequency (0.1 to 0.04%), both in vitro and in vivo under laboratory conditions (8, 12, 14).

Efficacy of chemotherapy and vaccination. Studies were done to determine whether vaccine administered at the time of contact would prevent transmission of the amantadine-resistant strains. Vaccine alone did not reduce mortality among the contacts, but when vaccine and amantadine treatment were administered simultaneously none of these birds died and they all developed antibody (Table 3). The dose of vaccine required to protect the birds was high; 9 or 18 μg of HA protein per dose plus 0.01% amantadine completely protected the birds, whereas lower doses of vaccine (3 μg of HA per dose) protected only half the birds. The dose of vaccine required to protect chickens (9 to 18 μg of HA per dose) under these conditions was of the same order as that required to protect humans (8 to 15 μg per dose [16]). Birds treated with vaccine alone or vaccine plus amantadine developed nearly identical levels of antibodies, indicating that amantadine does not interfere with the development of antibodies (Table 3).

DISCUSSION

In the initial studies, amantadine and rimantadine showed considerable promise as prophylactic agents in the treatment of highly virulent Chick/Penn influenza virus. However, under conditions simulating natural transmission of the virus, amantadine-resistant mutant viruses arose within 9 days; all of the contacts were infected and approximately 50% died. Simultaneous administration of H5N2 vaccine and amantadine protected the birds from infection, whereas vaccine alone did not. The efficacy of vaccine plus chemotherapeutic agent can be explained by the rapid response of chickens to vaccination with influenza virus. By the time amantadine-resistant viruses appeared (approximately 9 days after initial infection), the birds were immune. These results indicate that after the appearance of a highly virulent influenza virus outbreak, amantadine plus vaccine would be a viable option, especially under conditions when eradication is not a viable option.

Amantadine is believed to inhibit influenza virus replication by interfering with an early stage of virus growth; uncoating (9) and transcription by the polymerase of the infecting virus particles are prevented (17). It is possible that amantadine inhibits replication by raising the pH of the endosome and preventing the conformational change in the HA that is necessary for fusion (18). On the other hand, resistance has been associated with the matrix protein (12) and can be segregated independently of the HA and neuraminidase (1, 12), indicating that further studies are needed on the mode of action of this drug (17). Continued shedding of high doses of virus (up to $10^{7.3}$ EID_{50}/g) in the feces of amantadine-treated birds (which were infected 2 days before administration of the drug) also suggests that amantadine has other modes of action; this agent was surprisingly efficacious when used therapeutically.

It is probable that another outbreak of highly virulent influenza will occur in humans; the last severe outbreak occurred in 1918 to 1919. The question of the value of chemotherapeutic agents arises in the face of such an epidemic. Although amantadine-resistant viruses have been isolated with a high frequency in laboratory studies, resistant

viruses have not been reported in human trials. Although the site of influenza virus replication in avian species (intestinal) differs from that in mammals (respiratory), the possibility exists that during a severe epidemic of influenza, resistant strains might emerge and cause disease as they did in the present study. The high rate of emergence of amantadine-resistant strains would be a great disadvantage, but at this time these drugs offer the only possible approach to controlling a highly virulent new human influenza virus strain. If the chemotherapy-plus-vaccine approach is to be considered for the control of highly virulent influenza virus in humans or lower animals, it would have to be initiated before drug-resistant viruses reached an epidemic level; otherwise, the approach would have no advantage over vaccine alone.

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