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EVALUATION OF ANTIVIRAL COMPOUNDS AGAINST AVIAN INFLUENZA

by

Evan W. Call

A thesis submitted in partial fulfillment
of requirements for the degree

of

MASTER OF SCIENCE

in

Bioveterinary Science

UTAH STATE UNIVERSITY
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1991

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ABSTRACT

Evaluation of Antiviral Compounds Against

Avian Influenza

by

Evan W. Call, Master of Science

Utah State University, 1991

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Department: Animal, Dairy and Veterinary Science

Tests in vitro for antiviral activity against avian influenza viruses, A/Turkey/Sanpete/85 (H6N8) and A/Turkey/Sanpete/86 (H10N9), isolated in Sanpete County, Utah, utilized known antiviral agents, amantadine•HCl (adamantanamine hydrochloride) and ribavirin (1-ß-D ribofuranosyl-1,2,4-triazole-3-carboxamide). The testing involved evaluation of seven drug concentrations. Maximum tolerated dose, minimum inhibitory concentration and therapeutic indexes were determined for each drug used. Both drugs demonstrated reasonable antiviral activity. Then they were tested against the H10N9 strain in 10-day-old Broad White turkey poults. Large- and small-particle aerosol delivery systems were the delivery methods utilized. Various parameters, including water and feed consumption, health score (based on visible signs), recoverable lung virus, lung hemagglutination titer, and visual lung scoring were considered for the quantification of viral infection and, thus, antiviral activity in birds. Recovery of viable virus from infected turkey poults proved sporadic. Visible signs were varied and mild, limiting the usefulness of health scores. The onset of illness was reflected in the decline in water

consumption. Moderate antiviral activity was demonstrated with the use of ribavirin small-particle aerosol, but ribavirin fog was not effective. Amantadine produced slight antiviral activity when used with both delivery systems. The potential efficacy of both drugs and application methods indicate further study of these control systems is warranted.

(72 pages)

INTRODUCTION

The poultry industry is a major portion of the agricultural economy in the United States and has a large impact upon all of us, especially at the breakfast and dinner table. Information gathered in 1980 indicates that chicken is the second most-ordered restaurant meal (Lasley 1984). The yearly consumption of eggs is 265 per capita. Broiler consumption per person is 48.6 pounds, and the average person consumes 10.7 pounds of turkey per year (Lasley 1984). When these levels of consumption are compared, using the 1967 dollar standard, today's prices are less than one-half those of 25 years ago (Lasley 1984).

Avian influenza (AI) has become a major concern to the poultry industry over the last few years. Due to the trend toward farms with large numbers of closely confined birds (intensification), control of readily transmissible diseases such as AI is a critical concern.

The impact of avian influenza on the poultry industry can be devastating. In the October, 1983, through February, 1984, outbreak in Virginia alone, losses and depopulation totaled 65 flocks with 1,241,746 birds at a cost of $9,446,549 (Van Buskirk 1984). Estimates of total losses experienced by the poultry industry in the affected states include 17 million birds at a cost of approximately $61 million (Webster et al. 1985).

The current policy of the United States Department of Agriculture (USDA) in the control of pathogenic AI is the depopulation of flocks infected with the disease. There is some evidence, however, that depopulation did not stop the spread of the virus in the Virginia outbreak (Van Buskirk 1984). This also seems to be the case in the 1985 outbreak in Sanpete Valley, Utah, where affected and neighboring flocks were processed to depopulate the

immediate area of the outbreak. Despite these efforts, the disease continued to spread to other flocks throughout the valley (Ahmed and Jensen 1987). This justifies the necessity of examining other control alternatives.

Due to the lack of efficacy of the current control measures in practice, we examined the in vitro and in vivo efficacy of treating avian influenza infections with amantadine and ribavirin, two drugs known to be inhibitory to the influenza virus (Dolin et al. 1982; Khare et al. 1973; Oxford 1975a; Oxford 1975b; Sidwell 1980)

Initial experiments were conducted to demonstrate the activity of these compounds against recently isolated influenza viruses, in cell culture utilizing primary chick embryo fibroblast (CEF) cells; studies then were performed to examine the in vivo treatment of young (10-12 day old) turkey poults infected with the virus. Because of the ease with which large numbers of birds can be treated via aerosol, and the current ready availability of large-particle aerosol systems on many farms, we treated infected birds in separate experiments with either amantadine or ribavirin, utilizing fogs generated by a commercially available fogger.

It already has been demonstrated that ribavirin and amantadine are particularly efficacious when administered via small-particle aerosol (mean particle diameter <5 μm) (Knight et al. 1980). Therefore it was of interest to treat young infected turkey poults with small-particle aerosols of amantadine and ribavirin. We used a collison-type nebulizer built by the U.S. Army and powered by compressed air.

REVIEW OF LITERATURE

Characteristics of Avian Influenza

AI was first described in 1887 in Italy (Stubbs 1965) by Perroncito as "a serious disease of chickens." It was not until 1955, however, that the responsible pathogenic agent was found to be the type A influenza virus (Bankowski and Samadieh 1980).

Recent AI outbreaks have been unique in several ways. The 1983-1984 Virginia epizootic is the first major outbreak in chickens since 1929 (since 1960 the disease has been primarily a problem in turkeys); it produced a fowl plague-like disease, with high mortality that was reproducible in the laboratory (Beard 1984). Dr. C. W. Beard (1984) stated that:

> Prior to these isolations it was difficult if not impossible to take influenza virus from a disease outbreak in the field, take it to the laboratory and produce disease, especially high mortality where 23 of 25 or 24 of 26 SPF hens would die of the laboratory infections.

AI recently has become a disease of importance also in the state of Utah. In Sanpete Valley of central Utah late in 1986, 70-80% of the turkey flocks "had or were experiencing AI" (Ahmed and Jensen 1987). Mortality rates were as high as 20-30% in 4-7-week-old poults, where Escherichia coli complicated the infection.

Part of the difficulty inherent with diagnosis of AI is the broad spectrum of signs or lesions that are often seen with outbreaks of infections caused by this virus. Signs of the infection may be indistinguishable from Newcastle disease (U. S. Department of Agriculture 1984), and, in fact, in 1964 an outbreak of what was believed to be Newcastle disease (which also is a myxovirus or hemagglutinating virus) occurred in turkeys in Modesto, California. After careful study, however, the virus isolate was found to be an

influenza virus (Bankowski and Samadieh 1980). Signs of the disease were so varied that

> some strains of the virus may cause high mortalities in birds of any age while others may be of a form so mild that infection may go undetected (Bankowski and Samadieh 1980).

Even upon examination at necropsy, the disease signs are not consistent, nor particularly pathognomonic (Eckroade 1984). Possible signs can include depression and droopiness, ruffled feathers, loss of appetite, sudden drop in egg production, loss of coordination in walking and standing, cyanosis of comb and wattle or snood, diarrhea, blood tinged discharge at the nares, circling and paralysis. Upon necropsy, hemorrhages on the mucosa or lining of the ventriculus, proventriculus, and cardiac muscle may be seen, as well as necrotic lesions in the liver, spleen and kidneys; enlarged spleen; and pneumonic lungs. The development of AI is within 3 to 7 days of exposure, and death normally follows the onset by 2 to 4 days (U. S. Department of Agriculture 1984).

Cultivation of Avian Influenza Virus

Influenza virus is readily grown in hens' eggs and, with more difficulty, in cell culture (Dowdle and Schild 1975); however, eggs still are the cultivation system of choice for particular applications such as preparation of large virus pools and vaccine production (Murphy and Webster 1985).

Many influenza viruses can be readily propagated in cell culture either in primary CEF cells alone or in one of many other cell lines with addition of trypsin (Appleyard and Maber 1974; Beare and Keast 1974; Klenk et al. 1975). These other cell lines would include monkey kidney, calf kidney, primary bovine kidney, hamster kidney, chicken kidney and human embryonic lung

cells (Beare and Keast 1974; Gaush and Smith 1968; Murphy and Webster 1985).

It is thought that the ability of influenza virus to infect a cell is dependent on the fusion peptide found in the hemagglutinin (HA) molecule. This peptide, however, is not in a readily accessible antigenic site and a pH-induced conformational change is required in order for this fusion portion of the HA to become functional. This conformational change enables the HA molecule to bind to lipid vesicles and to hemolyze erythrocytes (Murphy and Webster 1985).

The exposure of F peptide of the HA also may be accomplished by trypsinization of HA molecule which has been shown to enhance viral infectivity. Tobita et al. (1975) found that 11 different influenza A viruses demonstrated increased plaquing efficiency with well-formed and enlarged plaque size due to addition of the proteolytic enzyme trypsin. This effect was seen at 5 μg/ml and demonstrated a dose response curve through 20 μg/ml, beyond which point no further enhancement was seen.

The treatment of influenza virus with a concentration of 10 μg/ml of trypsin caused the formation of plaques in CEF cells at or near the same level of infectivity as shown in 10-day chicken embryos for 6 strains of influenza A viruses tested (Appleyard and Maber 1974; Beare and Keast 1974). The titration of egg-grown virus in MDCK (Madine Darby Canine Kidney) cells revealed enhancement of plaqueforming infectivity from one-fold for influenza CK/Germany/34 virus to 1500-fold for influenza virus strain pt/N.I./73 with trypsin treatment (Alexander, Collins and Parkinson 1981).

The use of trypsin in overlay medium has extended the range of susceptibility of MDCK cells to include viruses that otherwise cannot be

assayed in this system, such as influenza AO/PR8, A1/CAM and A/swine (Tobita et al. 1975).

Tobita and Kilbourne (1974) found in cell culture under fluid maintenance medium without trypsin, using low multiplicity of infection, there was no evidence of virus growth, indicating an abortive infection. The inclusion of 2 μg of trypsin per ml in the fluid maintenance medium of primary CEF cells infected with influenza B/Lee/40 yielded $10^{7.0}$ 50% egg infective dose (EID_{50}), whereas without trypsin the titer was $10^{4.3}$ EID_{50}. Similar results also were demonstrated with influenza B/Mass/1/71.

Each virus strain varies from others in their plaquing response to the trypsinization. This variation is seen in virus titer and in plaque morphology (Appleyard and Maber 1974; Beare and Keast 1974).

The difference in response to effects of trypsin among the various strains of influenza A has also been seen with the A/WSN strain and with fowl plague virus, both of which produced good plaques with, and without, trypsinization. The A/Sing/1/57 required the presence of trypsin to even show development of plaques (Appleyard and Maber 1974).

Epidemiology or Transmission of Avian Influenza Virus

Avian influenza appears to be more readily transmissible than Newcastle disease. It is very difficult to control spread of the virus. Ninety-seven percent of the 1983-1984 outbreaks that occurred among chickens in Maryland, Pennsylvania, Virginia and New Jersey took place after it was publicized that a highly pathogenic AI virus had emerged. It was after November 9, 1983, when the extraordinary emergency was declared and

quarantine and eradication programs were initiated, that 77% of all outbreaks in the above states developed (Craig 1984).

In natural infection and chemotherapy studies reported by Lang, Narayan and Rouse (1970), uninfected turkeys were housed in the same pen as birds experimentally infected with a highly pathogenic strain of AI (A/Turkey/Ontario/66). Of the original uninfected birds exposed, all not treated with the antiviral agent amantadine died of avian influenza A contracted by simple cohabitation with infected birds (Lang, Narayan and Rouse 1970).

Westbury, Turner and Amon (1981) also showed evidence that the birds must come in contact with each other in order for the virus to be effectively transmitted. They did this by placing infected and uninfected birds in contact on the floor of an isolator house and by also suspending uninfected birds in cages, one bird's height above the floor containing infected birds. Under these circumstances, none of the caged birds became infected (Westbury, Turner and Amon 1981). This failure to transmit infection to birds not in direct contact may have been due to an AI strain variation or because the birds were housed in a tiled room which was washed out daily. This practice would eliminate virtually all the dust which, in an on-farm situation, could serve as a vector in the transmission of the virus.

The 1983-84 outbreak seemed to contradict the idea that birds must come in direct contact for transmission to occur. This recent outbreak behaved differently from previous outbreaks in that this outbreak was very difficult to contain, whereas previous outbreaks tended to be self-limiting due to their lower transmissibility (Beard 1984). This was evidenced by the fact that more than three-quarters of all cases of flock infection occurred after institution of quarantine and other control measures, such as the restriction of sale of contaminated birds and depopulation (Craig 1984; Webster et al. 1985). This

suggests the potential importance of feral and wild avian species in the transmission of this virus. Another possibility is human workers (catching crews and other service crews) functioning as a vector, as was suggested by the work of Campbell, Webster and Breese (1970) and Webster, Geraci and Petursson (1981).

Avian Influenza Virus Morphology

The influenza virus itself is approximately 80-120 nm in diameter and is covered with antigenic projections, or spikes, on the surface. There are 2 different types of surface antigens, the first being the HA antigen, which is approximately 6 times more numerous than the second antigen, neuraminidase (Joklik 1985). The virus is an enveloped, RNA-containing virus whose genome is divided into 8 segments. The information in Table 1 has been reproduced from Joklik (1985) and describes each gene and the protein it encodes.

Of particular interest are genes 4 and 6 (Table 1); gene 4 codes for the two-part HA protein spike that is responsible for attachment at infection. The fusion protein is a subprotein of the HA spike. The protein coded by gene 6 is the neuraminidase protein, also a surface antigen that is used in identification of influenza viruses.

There are 3 types of influenza: types A, B, and C. Type A is the only one known to have varying HA and neuraminidase. There are 13 HA antigens and 9 neuraminidase antigens currently recognized (Joklik 1985).

Table 1.--Influenza Virus Genes, the Proteins Encoded and the Protein Function

Gene	Protein	Protein Function
1	P1	Initiation of transcription
2	P2	Cap-binding protein
3	P3	Elongation of transcription
4	HA1	Large portion of HA spike
	HA2	Smaller portion of HA spike
5	NP	Nucleocapsid structural protein
6	NA	Neuraminidase
7	M1	Matrix protein
	M2	Nonstructural, unknown function
8	NS1	Nonstructural, unknown function
	NS2	Nonstructural, unknown function

from Joklik (1985)

Chemotherapy of Avian Influenza

The number of AI outbreaks taking place during the most stringent eradication and control efforts makes it quite evident that these methods alone are not enough. Thus we felt it important to investigate chemotherapy as an alternative.

Chemotherapy of viral diseases is much more complicated than treatment of bacterial infection. The obligate intracellular parasitic nature of viral infections is the major complicating factor, in that the invading virus utilizes the metabolic, protein and nucleic-acid duplicating mechanisms of the cell (Joklik 1988). This intimate intracellular relationship between virus and host cell makes extremely difficult the task of hitting a specific antiviral target while avoiding adversely affecting the host cell (Robins 1986).

Current antiviral chemotherapy efforts are directed at one or more stages of virus infection and replication. One likely target point in this cycle is the virus attachment to, and penetration of, the cell wall. Drugs designed to block or occupy the virus-binding sites on the cytoplasmic membrane would accomplish this (Oxford 1975b).

Another target in this cycle would be prevention of the uncoating or release of the influenza RNA into the cytoplasm of the cell. This mode of action has been shown to be how amantadine and rimantadine exert their prophylactic activity (Bukrinskaya, Vorkunova and Narmanbetova 1980).

The next logical step would be to inhibit one or more virus-induced or virus-enhanced metabolic activity or biochemical pathway within the cell. The nucleosides or nucleoside analogs, trifluridine, vidarabine, acyclovir, bromovinyl deoxyuridine, deazaadenosine and ribavirin, all are drugs whose efficacy falls into this category (Sim and McCullagh 1985). Of these inhibitors, only ribavirin is effective against influenza.

Other important targets for antiviral chemotherapy research include viral protein maturation, viral capsid assembly, genome packaging, and, for enveloped viruses like AI, budding (Sim and McCullagh 1985).

There are a number of agents that have been shown to have antiviral activity (Sidwell 1986). See Table 2 for a summary of some important clinically active antiviral drugs. Of particular interest are the first 3 compounds, all of which have been shown to be active against influenza A viruses.

A major difficulty that can arise in antiviral chemotherapy research is the occurrence of drug-resistant virus mutants, such as has been shown to occur with amantadine and its derivatives (Beard, Brugh and Webster 1987; Heider et al. 1981; Webster et al. 1985). The occurrence of drug-resistant mutants

Table 2.--Representative Clinically Active Antiviral Drugs

Drug	Chemical Name	Viral Disease Inhibited
Amantadine•HCl	1-Adamantanamine•HCl	Influenza A
Ribavirin	1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide	Hepatitis, influenza A and B, respiratory syncytial disease, measles
Rimantadine•HCl	Methyl-1-adamantanamine•HCl	Influenza A
Acyclovir	9(2-Hydroxyethoxymethyl)guanine	Herpes eye, cutaneous and genital infections
Cytarabine	1-ß-D-Arabinofuranosylcytosine	Herpes eye, cutaneous infections
Idoxuridine	5-Iodo-2'-deoxyuridine	Herpes eye,cutaneous and encephalitis infections
Methisazone	1-Methylisatin-3-thiosemicarbazone	Smallpox, severe vaccinial infections
Trifluorothymidine	5-Trifluoromethyl-2'-deoxyuridine	Herpes eye infections
Vidarabine	9-ß-D-arabinofuranosyladenine	Herpes eye, encephalitis infection
Zidovudine	3'-Azido-3'-deoxythymidine	Acquired immune deficiency syndrome
Ganciclovir	9-(1,3-dihydroxy-2-propoxymethyl)guanine	Cytomegalovirus infections

raises a special interest in the antiviral agent ribavirin. Ribavirin has a broad spectrum antiviral activity which includes a strong efficacy against influenza viruses (Sidwell 1980). Of more particular interest in this case, however, is the fact that no drug-resistant mutants have ever been found with ribavirin (Robins 1986).

Amantadine (1-adamantanamine hydrochloride). Amantadine is a cyclic primary amine (Figure 1) that has been shown to be effective against most influenza A virus infections in many animal species as well as humans (Hoffmann 1973). It was approved by the U.S. Food and Drug Administration for use in treatment of Asian influenza (H2N2) in humans in 1966 and in 1976 for use against all influenza A infections (Sim and McCullagh 1985).

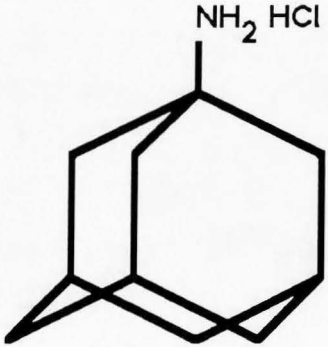

Fig. 1. Structure of amantadine.

At least 2 mechanisms of action of amantadine are known, which are dependent on drug concentration. At high concentrations (0.5 mM), amantadine increases the pH in the endosome containing the virus particle, stopping fusion of the virus and endosomal membranes due to the inhibition of the pH dependent HA configuration change, thus preventing the release of the nucleocapsid into the cytoplasm (Burkinskaya, Vorkunova and Narmanbetova 1980).

The low concentration inhibition of influenza A viruses by amantadine occurs early for human viruses and late for 2 different avian strains. This early vs. late inhibition is dependent on the HA present and maps to the M_2 gene (gene coding for a matrix protein). This suggests that amantadine may interfere with a HA-matrix protein interaction (Field and Owen 1988).

The first to demonstrate the susceptibility of AI in vivo to a chemotherapeutic agent was Lang, Narayan and Rouse (1970), who was able to prevent infection and subsequent death of turkeys with oral administration of amantadine. In their studies, 5 mg amantadine per kg body weight showed good prophylactic activity. Webster et al. (1985), working with chickens, found that levels higher than this up to 0.5% amantadine in the drinking water, were not well accepted. The birds treated at this level suffered reduced weight gain and in both experiments were susceptible to AI infection at the termination of treatment. However, at the 0.01% level it was found both amantadine and rimantadine were well tolerated, with no decrease in weight gain. Both drugs were effective at this level in preventing clinical signs, as well as infection, as demonstrated by the fact that only 1 of 10 rimantadine-treated birds sero-converted. At treatment concentrations of 0.002% all birds became infected, 1 of 10 died and all others showed signs of infection and recovered with levels of HA inhibition (HI) antibody with titers >640.

It is important to note that the same researchers found that an amantadine-resistant virus developed during simulated flock treatment and natural transmission caused mortality among treated birds (Webster et al. 1985). This was confirmed by Beard, Brugh and Webster (1987), who showed that 67% of 10-day-old embryos infected with the resistant virus in the presence of 400 mg amantadine/egg died.

Karunakaran (1984) studied the effect of treating AI infected turkeys with amantadine administered by oral gavage, drinking water, or as an aerosol. In the 5-week-old turkey poults treated with 20 mg/kg/day amantadine once daily for 6 days beginning 3 days pre-virus exposure, no recoverable virus was shed. Karunakaran (1984) also reported rimantadine to have moderate post-infection treatment efficiency whereas amantadine did not.

In birds receiving amantadine via aerosol twice a day for 3 days, lung tissue from 13% of the treated birds yielded recoverable virus, while 33% of untreated birds produced recoverable virus which had titers of approximately 10^6 virus particles per gram of tissue (Karunakaran 1984).

Another parameter of interest in the study of influenza infections is the reduction of water consumption. Using mice, McGahen and Hoffmann (1968) showed the reduction of water consumption in infected animals. The degree of water consumption reduction was correlated with amount of virus given in the inoculum and coincided with the severity of the disease (McGahen and Hoffmann 1968). With decrease in water consumption as the gauge of the onset of illness, McGahen and Hoffmann (1968) began amantadine treatments, via oral gavage and drinking water, 12 hours following the onset of reduction of water intake. Treated animals demonstrated significant reduction in disease severity, increase in the percent of animals remaining healthy, and lengthening of mean survival time. It is assumed, then, that the

water intake, or the absence thereof, could be used as a measure of prophylactic or efficacious treatment activity.

Because of the moderately positive effect of aerosol treatment we felt it important to study efficiency of aerosol treatment of amantadine and ribavirin, and to compare the relative effectiveness of large- (fog) and small-particle aerosols.

Ribavirin (1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). Ribavirin is a synthetic nucleoside that resembles guanosine (Figure 2). It has a truly broad spectrum of activity including inhibition of both RNA and DNA viruses (Oxford 1975a, Sidwell 1980, Sidwell et al. 1972).

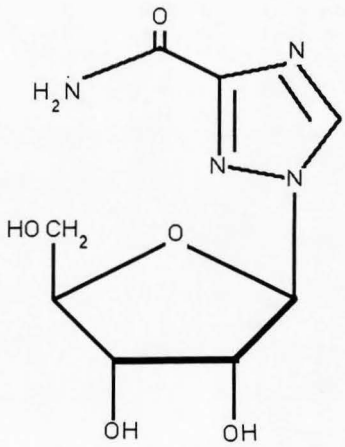

Fig. 2. Structure of ribavirin.

The precise mechanism of action remains partially unclear because no drug resistant mutant viruses have been found to help elucidate the mechanism of action. It is believed that this is due to ribavirin's having many sites of action (Robins 1986).

The mechanism of action of ribavirin and its metabolites is known to be multi-faceted. Initially, ribavirin monophosphate was shown to be a potent inhibitor of inosine monophosphate (IMP) dehydrogenase, in both cell-free (Streeter et al. 1972; Streeter et al. 1973) and viable-cell systems (Smith et al. 1974), which subsequently inhibited viral nucleic acid synthesis by limiting production of guanine nucleotides available for that synthesis (Streeter et al. 1977). Other researchers (Barnett, Shipman Jr. and Drach 1980; Drach, Thomas and Shipman Jr. 1978; Drach et al. 1981) using [^{32}P] uptake rather than the [^{3}H] used by Streeter et al. (1977) showed only slight effect on DNA synthesis, and marked inhibition of phosphorylation of [^{3}H] thymidine, thus suggesting the actual inhibition of DNA is less than had been assumed.

Sidwell, Revankar and Robins (1985) suggest that the mechanism of action of ribavirin must not be dependant on the effect on RNA and DNA synthesis because of the number of viruses against which ribavirin exerts little or no effect (poliovirus, coxsackie B virus and pseudorabies virus as well as adenovirus and rhino to varying degrees).

Eriksson et al. (1977) reported that in cell-free systems, between 82 and 90 percent inhibition of influenza virus RNA polymerase by ribavirin-triphosphate at the 500 μM concentration. The triphosphate form of ribavirin has been reported by Miller et al. (1977) to be found in cells at levels similar to those of ribavirin-monophosphate. However, ribavirin and ribavirin-monophosphate do not show inhibitory activity against influenza virus RNA polymerase (Eriksson et al. 1977). It is important to note that stimulation of

influenza virus cRNA (Plotch and Krug 1977) can be abolished by ribavirin-triphosphate (Eriksson et al. 1977).

The efficiency of translation of mRNA grown in the presence of ribavirin is greatly reduced (Canonico et al. 1980). Smith et al. (1980) and Goswami et al. (1979) found that ribavirin exhibited a potent effect on the 5' terminal guanylation of in vitro synthesized uncapped mRNA of vaccinia virus, and ribavirin may be incorporated into the cap (Smith et al. 1980). This is further supported by the fact that ribavirin is poorly active against poliovirus which does not have the normal 5' cap. These effects on viral mRNA may explain, in large part, ribavirin's antiviral activity.

Treatment of viral infections with ribavirin aerosols has been effective against several viral infections (Taber et al. 1983). Currently ribavirin is U. S. Food and Drug Administration approved for aerosol treatment of respiratory syncytial virus infections in infants.

Treatment of influenza A infections in mice with aerosols of ribavirin has demonstrated therapeutic activity (Stephen et al. 1976). Stephen and associates showed ribavirin to be effective when administered as late as 3 days post-infection; however, percent survivors increased from 53% to 93% when periodic daily treatments began 6 hours post-virus exposure. Extensive work using ribavirin has been done with influenza A infections in mice (Durr, Lindh and Forbes 1975; Khare et al. 1973; Stephen et al. 1976) and ferrets (Schofield et al. 1975) with various treatment routes, but work with ribavirin in turkey poults has not been reported. Karunakaran (1984) does report some preliminary work using ribavirin to treat influenza virus infections in chickens.

MATERIALS AND METHODS

Virus

Influenza A/Turkey/Massachusetts/65 (H6N12) (A/Turkey/Mass) was obtained from the American Type Culture Collection, Rockville, Md.

Influenza A/Turkey/Sanpete/85 (H6N8) and A/Turkey/Sanpete/86 (H10N9), were collected and provided by Dr. Marcus Jensen of Brigham Young University, Provo, Utah.

The A/Turkey/Mass virus was inoculated into the allantoic cavity of 10-day-old embryonated hen's eggs and fluids were collected on the day of death of the embryo. Eggs were refrigerated prior to collection of fluids to reduce the amount of bleeding during collection.

Fluids collected on the various days of embryo death were pooled and frozen at -80°C. Aliquots then were thawed and titered via egg inoculation and by HA.

Virus pools were titered on the days they were collected, with the pool having the highest titer chosen for developing a larger virus pool. Eggs were infected as before and on day 3, when 8 of 10 eggs exhibited hemorrhage and decreased mobility, fluids were collected, centrifuged at 1800 rpm for 3 minutes, aliquoted and frozen at -80°C. This pool was quantified by HA and had a mean end point of 1:352. The EID_{50} was $10^{2.5}$/ml as determined by the Reed and Muench (1938) method. The cell culture 50% infectious dose ($CCID_{50}$) of the pool, as assayed in MDCK cells, was $10^{4.4}$ ml.

Both viruses isolated in Sanpete County were propagated in eggs. The H10N9 virus caused death earlier than the H6N8 virus. However, this pool was found to be contaminated with two different Staphylococcus and an

Aeromonas bacterial species. Antibiotic sensitivity tests showed that there was no single treatment that could eliminate all of the contaminating species.

To verify the source of contamination, a second pool was grown in eggs using the same H10N9 virus pool as inoculum. The fluids from the eggs in this pool were again plated on LB (Lowenstein Broth) agar (10 gm Bacto tryptone, 5 gm Bacto yeast extract, 5 gm sodium chloride, 13 gm agar/litter, pH adjusted to 7.5 with NaOH) and the same 3 bacterial colony morphologies were seen. A flask of primary chick embryo fibroblasts (CEF) cells were also infected with this seed stock; within 3 days the cells were dead and a grainy background of motile contamination was visible.

In an effort to make this pool usable because of its applicability to Utah agriculture, and its relatively more virulent nature, the virus pool was centrifuged at 2100 rpm for 5 minutes in an effort to eliminate cellular debris and most of the bacterial contamination. The pool then was treated with 1 mg/ml of lysozyme and 20 mg of ethylenediaminetetraacetic acid (EDTA)/ml. A 0.5 ml aliquot of the treated pool then was plated on each of 2 LB agar plates undilute. After this treatment there was only 1 bacterial colony found on 1 of the plates. The other plate was free of bacterial contamination. The contaminates were determined to be normal flora of the turkey respiratory tract; since the route of inoculation of birds was the respiratory tract, it was decided that this pool would be usable for antiviral drug tests in birds. After treatment, this virus pool's EID_{50} end point was 10^4 and the $CCID_{50}$ was $10^{5.3}$. It should be noted that neither this nor the other viruses used in this work caused experimentally-induced death in turkeys.

Birds

One-day-old Orrlop, Broad White turkey poults were obtained from the Moroni Feed Co. Hatchery (Moroni, Utah). These birds were culls or seconds provided for this research at no cost. Birds were culled as seconds when visual examination revealed anomolies considered to indicate reduced vigor in the on-farm environment. Examples of culling criteria could include size, swollen umbilicus or beak malocclusion. In each case, birds were examined and determined to be healthy and acceptably vigorous for virus titration and antiviral tests. Being seconds, hatchery personnel provided assurance that these birds were not vaccinated with anti-AI vaccines currently in use. Poults were brooded in electrical heated battery brooders at the Utah State University (USU) Poultry Farm. The temperature was regulated at 32.2°C for the first 5 days and then reduced 3-5 degrees every other day, so on day 11 the poults were accustomed to approximately 23.8°C temperatures. Then they were moved to isolation sheds on the USU Veterinary Science farm. Birds were fed #1 Starter Mash ad libitum, obtained from Moroni Feed Co. Terramycin (Oxytetracycline Hydrochloride)powder was mixed with the feed at the rate of 500 grams per ton, in order to limit the severity of secondary infections that often accompany AI infections. Water was provided ad libitum.

Housing

Poults held at the USU Veterinary Science Farm were housed in 61 cm high plywood boxes measuring 76 cm^2 with a chicken wire frame top, hinged so that access to the birds was through the top of the box. The boxes were bottomless and stood on the concrete floor. Approximately 3" of pine shavings were spread on the floor as bedding. Bedding was changed weekly

and all facilities were disinfected between groups of birds with sodium hypochlorite (Clorox™).

Quarantine Procedures

Personnel working with infected birds were subjected to stringent hygiene and infection control procedures. Persons who had come in contact with infected birds were not allowed access to the USU Poultry farm. All deliveries of birds or feed were made to a clean drop point. Personnel exposed to the AI-infected birds would then pick up delivires and deliver them to the AI facility. Personnel coming in contact with infected birds were required to wear surgical mask, gloves, disposable outer garment and rubber boots that remained in the contaminated isolation shed at all times. Upon leaving the contaminated shed, personnel were required to disinfect any articles being removed as well as exposed surfaces and hands. These precautions were necessary to avoid the possible spread of AI to uninfected control birds housed in the USU Poultry Farm.

Induction of Virus Infection

Infection of poults were accomplished by passing an oral gavage needle (10 ga.) through the larynx as it opened upon aspiration. Virus in a volume of 0.1 ml thus was deposited in the bronchi. Virus used in antiviral trials was A/Turkey/Sanpete/86 diluted to $10^{3.3}$ $CCID_{50}$/0.1 ml.

In indicated assays birds were infected via intranasal infection, by holding the poult upside down and opening the beak, exposing the nasal cleft. Virus inoculum in the amount of 0.1 ml was deposited in the sinus cavity using an oral gavage needle (16 ga.).

Drug Treatments

Four different antiviral drug trials were conducted, 2 using ribavirin and 2 using amantadine. Each of these drugs was administered via 2 different aerosol systems. The first utilized a collision-type small-particle aerosol generator (Figure 3) obtained from the U.S. Army, (Dugway Proving Grounds, Utah) facility. This device produced aerosol particles measuring approximately 1-5 μm in diameter. The aerosol was delivered into a stainless steel chamber where the birds were exposed for 3.8, 7.5, 15 and 30 minutes. The second aerosol system was a Fogmaster POW-R-JET™, Fogmaster Corporation, (Deerfield Beach, Florida). This fogger delivered an aerosol with particle sizes varying from 30 to 100 μm, into a lexan plastic chamber of identical dimensions as the stainless steel chamber (54.5 cm x 18.1 cm x 22 cm) used in previous experiments. The total dosage again was controlled by varying the treatment time. The length of treatment was varied to administer approximately the same volume of drug per treatment, whether by large- or small-particle aerosol. The drug preparations consisted of 20 mg/ml in sterile saline and approximately 52.5, 105, 210 and 420 mg were administered per cage per treatment. Treatments began 18 hours post-virus exposure using ribavirin and 24 hour previous to virus exposure when using amantadine. Treatments were given twice daily for 3 days. On day 4, half of the infected birds were sacrificed and lung tissues were collected and scored as described under Infection Parameters.

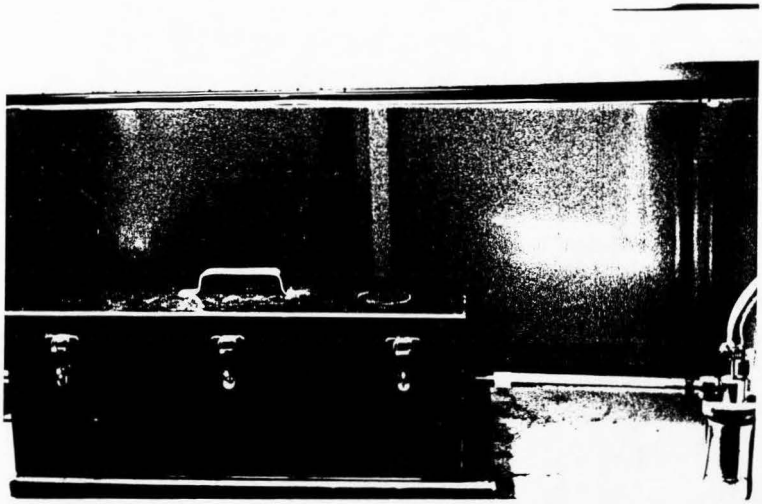

Fig. 3. Collision-type small-particle aerosol generator and chamber.

Infection Parameters

Health Score. Daily health scores were collected as an average of the birds in that cage, based on the presence of individual signs of disease, as follows:

0: Healthy, alert birds

1: Coughing, sneezing or other respiratory distress

2: Ruffled feathers, holding wings away from body

3: Droopy head, sinus exudate or swelling

4: Unwillingness to move, nesting down, stargazing

Birds were observed each day at the same time and individual bird scores were used to calculate an average cage score.

Lung Score. On day 4 of each experiment one-half of the infected and treated birds and 4 to 5 normal control birds were sacrificed and the lungs removed. The lungs were scored on a 0-4 scale based on the visual determination of percent of total area of the lungs that appeared consolidated (consolidation being defined as a reddening or darkening deviation from the normal salmon pink color of lung tissue, and firmness or loss of the spongy response to touch) as follows:

0: No consolidation

1: 25% consolidation

2: 50% consolidation

3: 75% consolidation

4: 100% consolidation

Water Consumption. Each morning the waterers were filled from a 1000 ml graduated cylinder and the volume of water required was recorded. A

decrease in water consumption has been reported to coincide with the development of influenza infection in mice (McGahen and Hoffmann 1968).

Recoverable Virus Titer. The tissue samples collected for lung scores were frozen at -80°C and later thawed and homogenized in either an Omni-Mixer homogenizer (Newton, Connecticut) or a Stomacher (Unique Scientific Apparatus, Cincinnati, Ohio). Tissues from the ribavirin-aerosol experiment were 15% weight/volume (w/v) homogenates; all other homogenates were 20% w/v, to conserve on homogenizing media and increase the possibility of virus isolation. Homogenizing medium was MEM (minimum essential medium) with 0.5% $NaHCO_3$, 1% sorbitol (Gibco Laboratories Life Technologies, Grand Island, New York). Tissues were homogenized for 1 minute, decanted to 5 ml snap cap vials and refrozen at -80°C. Samples later were thawed, diluted in serial 10-fold dilutions (diluted in MEM o.1%$NaHCO_3$, 1% sorbitol, 50 μg gentamicin/ml, without serum), and plated on 96-well microtiter plates (Titertek, Hayward, California), which had been seeded 24 hours previously with single passed CEF cells.

Weight Gain. Poults were weighed prior to infection and treatment and prior to sacrifice and tissue collection on day 4 post-virus exposure. Average weight gain or loss was used to indicate severity of infection and toxicity of drug treatments.

HA Titration. The same tissues used for recovered virus titer were used to do HA titrations using 0.3% suspensions of chick red blood cells (red blood cells provided by the USU Poultry Farm). A 0.1 ml of each sample was serially diluted 1:2 through 1:128 in phosphate-buffered saline (PBS). Red blood cell suspension then was added and mixed via repeated pipetting. The tests then were allowed to stand and readings were taken at 30 minutes and 2 hours. A solid button of sedimented red blood cells was read as a negative test. Any

disruption of the button formation was read as a positive test. The red blood cells were suspended in chilled (PBS).

Cell Culture. Primary CEF, MDCK and WI-38 (Human Embryonic Lung) cells were maintained in MEM with 5% fetal bovine serum (FBS) and 0.1% $NaHCO_3$ without added antibiotics, or Basil Minimal Eagles (BME) with 10% FBS, 0.1% $NaHCO_3$ (Gibco Laboratories Life Technologies, Grand Island, New York), in a 37°C incubator with 5% CO_2. The CEF cells were primary cultures generated by the trypsinization of decapitated 10-day-old chick embryos. Cells were grown for 3 to 4 days in Corning tissue culture flasks (Palo Alto, California). For cell culture titrations and antiviral tests, 96-well microtiter plates were seeded at 3.5×10^5 cell/ml with 0.2 ml/well. Virus dilutions and maintenance media for virus and trypsin titrations used appropriate cell culture media devoid of serum.

In Vitro Antiviral. In vitro antiviral tests were conducted utilizing the Sidwell and Huffman micro tissue culture antiviral test system (Sidwell and Huffman 1971). The 96-well flat-bottom plates were prepared with 24-hour-old confluent monolayers of single-passed primary CEF cells.

Drugs were diluted in MEM containing 0.18% $NaHCO_3$ and 50 μg gentamicin/ml but devoid of serum. Drug dilutions were made utilizing half-log dilutions starting at 1000 μg/ml through 1.0 μg/ml.

The growth media was splashed from the plate and 0.1 ml of each drug dilution was placed in each of 4 replicate wells, with dilution media placed on the virus controls. A 0.1 ml sample of the appropriate virus dilution then was added to each of 3 of the test wells and the virus controls; the fourth well received virus dilution media in order to serve as a toxicity control. The plates were checked 3 and 5 days later for adequate CPE development, and were read on day 5. Virus control and test well CPE were graded on percent basis

as described earlier. From this data virus rating (VR) (percent CPE of virus control - percent CPE of test/ 10 X number of test wells), effective dose 50% end point (ED_{50}) (obtained by plotting percent inhibition vs. drug dilution and estimating the 50% dose from line intersection), Cytotoxic dose 50% end point (CD_{50}) (visual determination of 50% toxicity in toxicity control wells) and therapeutic index (TI) (the ratio of CD_{50}/ED_{50}) were determined.

Titrations. Turkey lung and egg preparation samples were titered in 96-well microtiter plates seeded as above. Triplicate wells were inoculated with serial 10-fold dilutions. End points were determined via microscopic examination for visually recognizable CPE, and in some cases the CPE was verified by collecting supernatant from the last positive wells in the titration following a freeze-thaw process. These supernatants then were diluted for HA titration and positive HA used to indicate correct reading of visually recognizable CPE.

RESULTS

Development of a Cell Culture and Assay System for Avian Influenza Virus.

To establish cell line infectivity of these AI viruses, several experiments were conducted to establish which cell line or lines would be most susceptible to virus induction of CPE.

Since trypsinization of the viral HA has been shown to increase the infectivity of certain influenza viruses, the viruses available to us were therefore titrated in various cell lines at trypsin concentrations varying from 0 to 40 μg/ml (Table 3).

Both viruses isolated from Utah poults exhibited production of CPE in primary CEF cells without addition of trypsin; however, virus titers of the 1986 isolate increased with use of trypsin at 20 μg/ml. The 1985 isolate virus titer decreased by 0.5 $\log_{10}$ using moderate trypsin concentration. The only other virus showing a response to the trypsinization in CEF cells was the A/Turkey/Mass/65 isolate which had higher infectivity (0.2 $\log_{10}$), at the 40 μg/ml level of trypsin. None of the titer differences were significant statistically.

The A/Turkey/Mass/65 (H6N8) pool produced in eggs, reproduced modestly well in MDCK cells ($10^{4.4}$ $CCID_{50}$/0.1 ml) as determined by visual CPE detection, but did not produce readable CPE in MDBK cells beyond a 10^{-1} dilution. In 10-day-old embryonated chicken eggs, the virus titer was $10^{7.6}$ and the HA titer was $10^{2.7}$ (last dilution showing positive HA). Of particular interest, because of direct applicability to Utah agriculture, were the 2 viruses isolated in Sanpete County, Utah. The earlier isolate (A/Turkey/Sanpete/85 [H6N8]) had lower titers when grown in embryonated eggs as titered in CEF than the later viral isolate (A/Turkey/Sanpete/86

Table 3.--Effect of Trypsin Treatment[a] on In Vitro Virus Infectivity

	Virus Titer (log_{10} $CCID_{50}$)[b]					
Cell line:	MDCK	MDBK	MDCK	Primary CEF	Primary CEF	Primary CEF
Virus:	A/Tur/Mass	A/Tur/Mass	High Passage A/Tur/Mass	A/Tur/San/85	A/Tur/San/86	A/Tur/San/86 1 Turkey Passage
Trypsin Conc. ug/ml						
0	2.5	0	1.5	4.0	5.3	0
2	2.5	1.5	1.5	NT[c]	NT	NT
10	2.5	1.3	1.5	NT	NT	NT
20	2.5	1.3	1.5	3.5	6.5[d]	1.7
30	2.5	1.5	1.5	NT	NT	NT
40	2.7	1.5	1.5	NT	NT	NT

[a]Trypsin in the concentrations indicated was included in virus medium and subsequently added to the various cells and incubated with virus and cell until viral CPE was determined.

[b]Cell culture infectious dose 50% end point.

[c]NT= Not titered.

[d]Bacterial contamination found in this titration; therefore, the validity of this higher titer is questionable.

[H10N9]). The titers were $10^{4.0}$ and $10^{5.3}$, respectively in these cells. None of the AI viruses grew to appreciable titers in MA104 or L cells. Although they did reproduce slightly better in WI-38 cells, the titers still were too low to be useful.

Although the titer was slightly lower, more consistent CPE from the A/Turkey/Sanpete/85 (H6N8) virus was found in CEF cells. Antiviral tests using this system appeared more reliable than tests utilizing the later Utah isolate A/Turkey/Sanpete/86 (H10N9) due to inconsistency of infection in the virus controls.

Antiviral drug tests in vitro utilizing a one-half-log dilution scheme from 1000 μg/ml drug concentration to 1.0 mg/ml demonstrated good antiviral activity for both ribavirin and amantadine (Table 4).

Ribavirin was strongly active down to the 100 μg/ml treatment level with no cytotoxicity seen at the levels used in this test. The 50% effective dose (ED_{50}), determined by plating dose to percent inhibition, was 66 μg/ml.

Amantadine tested in vitro exhibited 75-100% toxicity at 1000 and 320 μg/ml; antiviral activity was seen at all lower dosages employed. The ED_{50} was determined to be approximately 8.3 μg/ml.

The percent toxicity and inhibition of AI in cell culture are summarized in Table 5.

Development of an In Vivo
Avian Influenza Test System

Inherent in the development of an in vivo system for AI was the verification of an applicable infection procedure. In such procedures the gross qualification of illness should be demonstrated in addition to the microbiological quantification of the severity of the illness.

Table 4.--Inhibition of Avian Influenza (A/Turkey/Sanpete/85 [H6N8]) Virus Infection in Primary CEF Cells by Ribavirin and Amantadine

Concentration µg/ml	Ribavirin			Amantadine		
	Percent[a] Toxicity	Avg. CPE	Percent[b] Inhibition	Percent[a] Toxicity	Avg. CPE	Percent[b] Inhibition
1000	0	0	100	100	Toxic	Toxic
320	0	12.5	83	100	Toxic	Toxic
100	0	21	72	75	25	67
32	0	75	0	50	25	67
10	0	87.5	0	25	38	49
3.2	0	96	0	0	38	49
1.0	0	100	0	0	58	23
0	-	75	-	-	75	-

[a]Percent toxicity is a visual determination based on a single microtiter 96-well sample at each concentration.
[b]Percent inhibition = virus control CPE - test CPE / virus control CPE.

Table 5.--Summary of the Inhibition of Avian Influenza A (A/Turkey/Sanpete/85 [H6N8]) Virus Infection in Primary CEF Cells by Ribavirin and Amantadine

	Ribavirin	Amantadine
CD_{50}[a]	1000 µg/ml	32 µg/ml
ED_{50}[b]	66 µg/ml	8.3µg/ml
VR[c]	>0.8	0.5
TI[d]	>15.2	<3.9

[a]50% Cytotoxic dose
[b]50% Effective inhibitory dose
[c]Virus Rating
[d]Therapeutic index

Infection with A/Turkey/Mass/65. Virus (A/Turkey/Mass/65) grown in eggs, when administered via the intranasal route, was found to establish a mild sinusitis in 21-day-old turkey poults. No deaths were observed. One bird infected with undilute virus developed a severe sinusitis on day 7 of infection, with sinus exudate, star-gazing (head tipped as though looking at the stars) and loss of equilibrium exhibited. Several birds developed swelling around the eyes. On day 4, however, following the initial development of signs, all clinical evidence of disease had abated. The disease signs were erratic and inconsistent, with no apparent infection dose response. Birds in all levels of infection (undilute through 10^{-4}, or $10^{4.4}$, $10^{3.4}$, $10^{2.4}$, $10^{1.4}$ $CCID_{50}$/0.1 ml) showed mild to no signs of clinical illness.

Infection with A/Turkey/Sanpete/86. In vivo titration of A/Turkey/Sanpete/86 in birds infected via laryngeal gavage also resulted in no deaths and only mild disease signs, with 62% of birds exhibiting indications of respiratory distress and sneezing. Lungs from these distressed birds showed highest consolidation at the $10^{-1.0}$ level of virus inoculum, which represents $10^{3.8}$ $CCID_{50}$ /bird. See Table 6 for virus inoculum, and resulting lung scores.

Infection with Turkey-passed A/Turkey/Sanpete/86. Lung homogenates collected on day 4 of infection from 10-day-old turkey poults infected with A/Turkey/Sanpete/86 was titrated in birds. This pool also produced no deaths, and only 2 birds of 51 showed any signs of disease. More profound in this case was an apparent infection dose-response in feed consumption. Although these differences in food consumption were not quantified, all feeders were filled to the same level and the relative volumes were recorded after 24 hours. This was done only once, at the peak of infection. Birds infected with virus diluted to 10^{-4} consumed approximately 90%; those at 10^{-3} consumed 100%, those at 10^{-2} consumed 75%; those at 10^{-1} consumed 60%, and birds infected

with undilute virus consumed 50% of the feed given. On day 5, one-half of these birds were sacrificed and the lungs scored. The lung scores demonstrated no significant differences between uninfected controls and all levels of infected birds. See Table 6.

Table 6.--Average Lung Score[a] of Turkey Poults Infected with Avian Influenza

Virus Strain	Normal Control	Virus Dilution[b]			
		10^0	10^{-1}	10^{-2}	10^{-3}
A/Turkey/San/86	1.9	1.8	2.9*	1.7	1.8
A/Turkey/Mass/65	1.8	1.1	1.6	1.4	2.0
A/Turkey/San/86/1t[c]	2.8	3.4	3.4	3.0	2.9

[a]Lungs collected on day 4 of infection and scored from 0 (no consolidation) to 4 (100% consolidation).
[b]Titer of virus strain used ($CCID_{50}$): A/Turkey/Sanpete/86 = $10^{4.8}$; A/Turkey/Mass/65 = $10^{4.4}$; A/Turkey/Sanpete/86/1t = not determined.
[c]Virus passed one time through turkey poults.
*$P<0.05$

Due to the relatively high lung scores in uninfected controls, other possible causes of consolidation were considered. A bacterial isolate was found in all lung samples tested. This will be discussed later.

Virus recovery from infected birds in these titrations was sporadic at the various levels of infection. Positive isolations were found as determined by viability in 10-day-old embryonated hens' eggs. Although these results seemed erratic, there was an apparent infection response curve (Table 7). One embryo death did occur in the uninfected controls; this is presumed to be due to technique, although this death occurred on day 5.

Table 7.--Egg Infectivity of Homogenates of Turkey Lungs Infected with Avian Influenza (A/Turkey/Sanpete/86)[a]

Lung Homogenate Dilution	Bird 1	2	3	4
10^{-1}	2/10	4/10	3/10	1/10
10^{-2}	1/10	0/10	0/10	0/10
10^{-3}	1/10	0/10	0/10	0/10
Uninfected lung	0/10			
Diluent control	1/6			

[a]Total embryo deaths 1/total embryos infected with lung homogenates.

In Vivo Drug Tests

Treatment of young turkey poults with a small-particle aerosol of ribavirin beginning 18 hours post-virus exposure (Table 8) showed a significant increase in water consumption at treatment durations of 30, 15, and 7.5 minutes. Increases in water consumption ranged from 10 to 14 grams above the control and lowest treatment group. Weight gain, however, was highest in the 7.5 minute treatment. This was the only significant weight gain in this trial. Lung HA titer also was significantly reduced at the 30-minute treatment group, showing a $\log_2$ titer of 1, while virus controls demonstrated a titer of 2.6. There also appeared to be tendency toward reduced lung score, although this parameter did not show significance at any treatment duration.

Similar treatment of infected poults with a large-particle aerosol or fog of ribavirin with treatments beginning 24 hours post-virus exposure did not cause significant differences between the 4 treatment groups and the untreated

Table 8.--Effect of Treatment of Avian Influenza Virus-Infected Turkey Poults with Small-Particle Aerosols of Ribavirin[a]

Treatment Duration (min/ treatment)	Total Ribavirin/ Cage (mg)	Toxicity Control		Infected, Treated			
		Mean Wt. Gain[b] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Wt. Gain[b,h] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Lung Score[d]	Mean Lung HA Titer[e] (Log_2)
30	420	125 ± 27.3	108 ± 5.15	140	104* ± 7.1	2.7 ± 0.5	1.0** ± 0.6
15	210	124 ± 23.2	112 ± 10.6	117	103* ± 8.2	2.0 ± 0.3	4.0 ± 0.7
7.5	105	125 ± 27.3	126 ± 12.4	145*	108* ± 14.3	2.1 ± 0.3	2.0 ± 0.7
3.8	53	126 ± 16.2	112 ± 15.7	129	83 ± 6.4	1.7[f] ± 0.4	3.0 ± 1.5
Normal Control	0	g		g		1.5 ± 0.6	0.2 ± 0.2
Virus Control					94 ± 8.7	2.9 ± 0.2	2.6 ± 0.6

[a]Treatment bid x 3 beginning 18 hr post-virus exposure; aerosol generated by a collison-type aerosol device.

[b]Difference between weights determined on Day 0 and Day 5.

[c]Determined on Days 1 through 6.

[d]Scores assigned from 0 (normal) to 4 (maximal) on lungs taken 4 days post-virus exposure.

[e]Viral HA determined on lungs taken 4 days post-virus exposure.

[f]Only 3 lungs scored at this treatment level compared to 5 in other groups.

[g]Normal control housed at separate facility; weight gain and water consumption data were therefore not gathered.

[h]Standard deviation of these data cannot be calculated due to unpaired nature of the data.

*$P<0.05$

**$P<0.01$

(Treatment groups n = 10 except 3.8 min. where n = 7. Control groups n = 5 except 3.8 min where n = 3.)

controls (Table 9). Mean weight gains remained constant except for birds in the shortest treatment duration group, where there appeared to be an appreciable increase in weight. This increase was not significant, due to a small sample size in the final weight average. Water consumption, mean lung score and lung HA titer showed very little or no variation.

Birds treated 24 hours pre-virus exposure with a small-particle aerosol of amantadine (Table 10) showed consistent water consumption, only varying from 91 to 101 ml/bird. Mean lung HA titers appeared to show a mild dose response curve with a mean titer of 2.1 (Log_2) at the 30-minute treatment duration and a titer of 3.3 at the 7.5-minute treatment duration. The 30-minute treatment group exhibited significant reductions in the mean lung score with an average score of 0.78, or approximately a 20% consolidation, while the infected untreated controls averaged approximately 33% consolidation. The use of a fog of amantadine to treat poults infected with AI beginning 24 hours prior to infection (Table 11) resulted in a uniform weight gain in all groups except the 1.5 second treatment, in which a significantly lower weight gain resulted. All the other treated and infected groups' weight gains were consistently lower than the virus controls by 6-7 gm. Likewise, the highest water consumption was in the virus controls; the lowest water consumption occurred in the 3-second treatment group with essentially a dose response seen. Mean lung HA titers also varied in an approximate dose response fashion, with the 2 significantly low titers seen in the 2 groups receiving the longest treatments. The highest HA titer was not in the virus control but in the shortest treatment group. Mean lung scores in this trial did not vary significantly in any of the treatment groups, with all the scores ranging between 2 and 2.5.

Table 9.--Effect of Treatment of Avian Influenza Virus-Infected Turkey Poults with a Fog of Ribavirin[a]

Treatment Duration (sec/ treatment)	Total Ribavirin/ Cage (mg)	Toxicity Control		Infected, Treated			
		Mean Wt. Gain[b] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Wt. Gain[b,g] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Lung Score[d]	Mean Lung HA Titer[e] (Log_2)
3.0	420	91 ± 14.3	98 ± 10.6	84	104 ± 8.5	1.3 ± 0.5	2.6 ± 0.5
1.5	210	85 ± 15.8	96 ± 8.8	81	94 ± 8.3	2.0 ± 0.4	3.2 ± 1.4
0.75	105	80 ± 9.2	80 ± 9.0	82	94 ± 8.3	1.1 ± 0.3	2.8 ± 1.1
0.38	52.5	82 ± 7.2	89 ± 18	117	98 ± 7.3	1.4 ± 0.3	3.0 ± 0.6
Normal Control	0	f	f			0.7 ± 0.2	3.0 ± 0
Virus Control	0			86	95 ± 4.7	1.7 ± 3.4	2.0 ± 0.9

[a]Treatment bid x 3 beginning 18 hr post-virus exposure; fog generated by a Fogmaster 6309 Power-jet fogging device.

[b]Difference between weights determined on Day 0 and Day 5.

[c]Determined on Days 1 through 6.

[d]Scores assigned from 0 (normal) to 4 (maximal) on lungs taken 4 days post-virus exposure.

[e]Viral HA determined on lungs taken 4 days post-virus exposure.

[f]Normal controls housed at separate facility; weight gain and water consumption data were therefore not gathered.

[g]Standard deviation of these data cannot be calculated due to unpaired nature of the data.

(Note: no statistical significance using any parameter. Treatment groups n = 10. Control groups n = 7 except 0.38 sec. where n = 6.)

Table 10.--Effect of Treatment of Avian Influenza Virus-Infected Turkey Poults with Small-Particle Aerosols of Amantadine[a]

Treatment Duration (min/ treatment)	Total Amantadine/ Cage (mg)	Toxicity Control		Infected, Treated			
		Mean Wt. Gain[b] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Wt. Gain[b] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Lung Score[d]	Mean Lung HA Titer[e] (Log_2)
30	420	106 ± 6.5	108 ± 8.0	nd[f]	91 ± 10.0	0.8* ± 0.3	2.1 ± .8
15	210	56 ± 16.3	115 ± 11.5	nd	100 ± 7.5	1.3 ± 0.8	2.9 ± .5
7.5	105	60 ± 20.0	111 ± 9.9	nd	92 ± 12.2	0.9 ± 0.3	3.3 ± .8
3.8	53	65 ± 5.2	118 ± 8.1	nd	97 ± 8.0	1.4 ± 0.3	
Normal Control	-	61 ± 9.7	105 ± 10.5			1.5 ± 0.3	4.0 ± 0
Virus Control				nd	108 ± 7.3	1.4 ± 0.4	2.6 ± .8

[a]Treatment bid x 3 beginning 24 hr pre-virus exposure; aerosol generated by a collison-type aerosol device.

[b]Difference between weights determined on Day 0 and Day 5.

[c]Determined on Days 1 through 6.

[d]Scores assigned from 0 (normal) to 4 (maximal) on lungs taken 4 days post-virus exposure.

[e]Viral HA determined on lungs taken 4 days post-virus exposure.

[f]Not determined.

*$P<0.05$

(Treatment groups n = 15 except 3.8 min. where n = 14. Control groups n = 6 except 30 min. where n = 5.)

Table 11.--Effect of Treatment of Avian Influenza Virus-Infected Turkey Poults with a Fog of Amantadine[a]

Treatment Duration (sec/ treatment)	Total Amantadine/ Cage (mg)	Toxicity Control		Infected, Treated			
		Mean Weight Gain[b,f] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Weight Gain[b,f] (g)	Mean H_2O Consumption[c] (ml/bird)	Mean Lung Score[d]	Mean Lung HA Titer[e] (Log_2)
3.0	420	85	125 ± 15	69	120* ± 12	2.1 ± 0.4	2.7* ± 0.5
1.5	210	69	127 ± 5	48	130 ± 8	2.5 ± 0.3	2.1* ± 0.6
0.75	105	92	162 ± 12	69	135 ± 12	2.1 ± 0.4	4.0 ± 0.5
0.38	53	88	162 ± 9	68	134 ± 7	2.0 ± 0.5	5.1 ± 0.2
Normal Control	0	105	172 ± 24				1.3 ± 0.1
Virus Control	0			75	153 ± 15	2.1 ± 0.3	4.3 ± 0.4

[a]Treatment bid x 3 begining 24 hr pre-virus exposure; fog generated by a Fogmaster 6309 Power-jet fogging device.

[b]Difference between weights determined on Day 0 and Day 5.

[c]Determined on Days 1 through 6.

[d]Scores assigned from 0 (normal) to 4 (maximal) on lungs taken 4 days post-virus exposure.

[e]Viral HA determined on lungs taken 4 days post-virus exposure.

[f]Standard deviation of these data cannot be calculated due to unpaired nature of the data.

*$P<0.05$

(Treatment groups n = 15. Control group 3.0 sec. n = 8, 1.5 sec. and normal n = 7, 0.7 sec. and 0.38 sec. were n = 6.)

Graphs of the per-day water consumption averages, per-treatment group, of birds treated with small-particle aerosol of ribavirin, yielded erratic results. The virus controls and the normal controls demonstrated the most linear results (Figures 4 and 5). It is of interest to note that all the treatment groups showed a downward spike at days 3 and 4. All groups, other than the 3.3-minute treatment and the normal controls, exhibited a general upward trend.

Similar data from trials where poults were treated with ribavirin fog demonstrated a more consistent upward tendency, with normal control and the 3-second treatment groups having the highest water consumption at day 7. The water consumption of all treatment groups remained fairly closely bunched until day 6, when they experienced a seemingly random divergence (Figures 6 and 7).

The graph of water consumption of poults treated with small-particle aerosols of amantadine (Figures 8 and 9) indicated wide rises and falls from day to day. From day 1 to day 2 a sharp, increase occurred, with an adjacent sharp valley at day 3; the least affected of the treatment groups at day 3 was the 15-minute treatment, wherein occurred the most stable increase over the 7 days. From day 3, all groups had a sharp increase in water consumption to day 5 or day 6, where they again exhibited declines to levels nearly equal to those seen at day 1 (Figures 10 and 11).

Water consumption data for poults treated with amantadine via fog demonstrated the most consistent linear results (Figures 10 and 11). Normal controls showed the highest water consumption overall with the only deviation being on day 4. In all treatment groups, water consumption remained closely grouped and linear through the 9 days of data collection, while both virus and normal controls both exhibited generally higher water consumption.

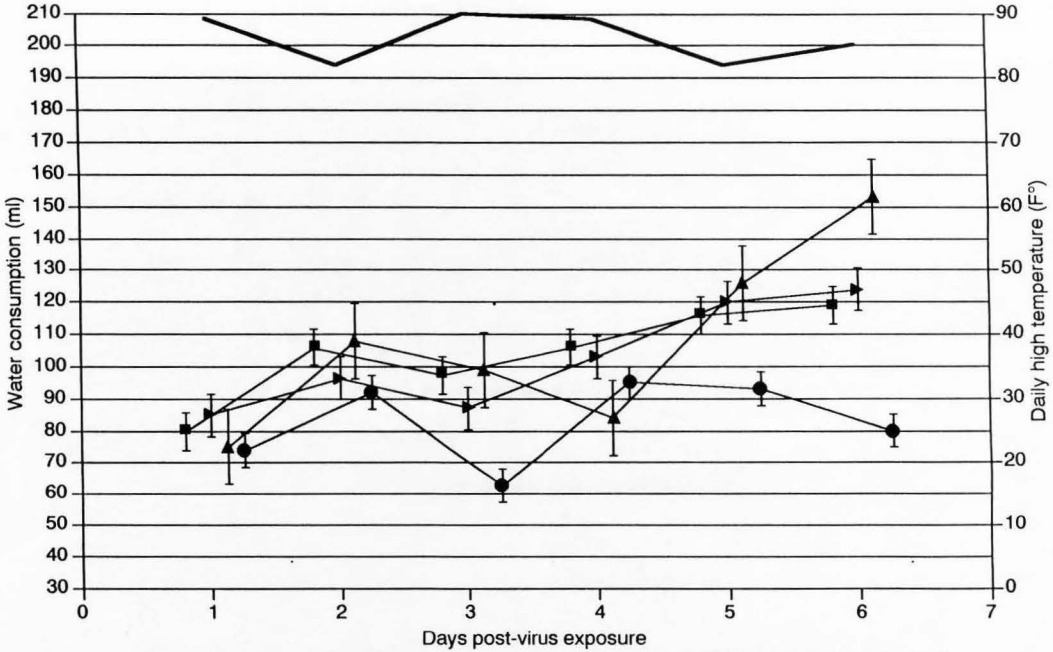

Fig. 4. Daily water consumption of infected poults treated with small-particle aerosol of ribavirin (bold line indicates daily high temperature F°). —■— 30 min. —▶— 15 min. —▲— 7.5 min —●— 3.3 min
—— Temp

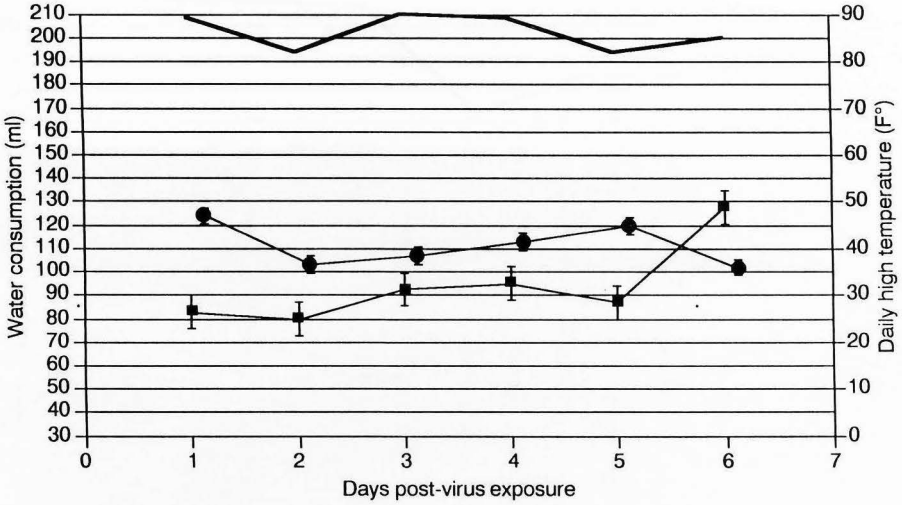

Fig. 5. Daily water consumption of uninfected control birds treated with small-particle aerosol of ribavirin (bold line indicates daily high temperature F°). —■— Virus Control —●— Normal Control —— Temp

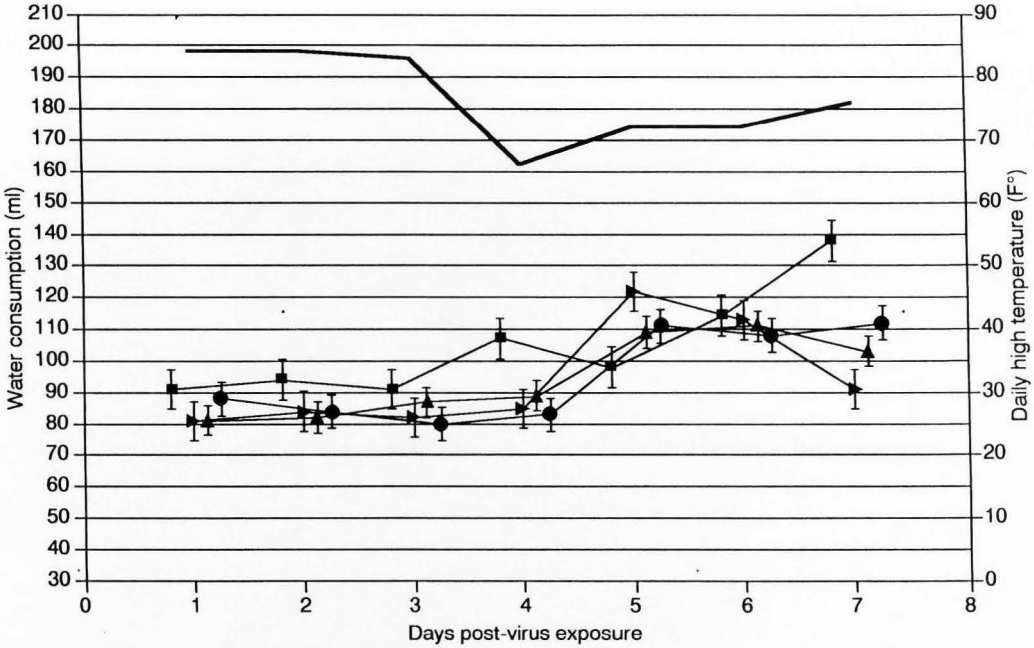

Fig. 6. Daily water consumption of infected poults treated with ribavirin fog (bold line indicates daily high temperature F°). —■— 3.0 sec —▶— 1.5 sec —▲— .75 sec —●— .38 sec —— Temp

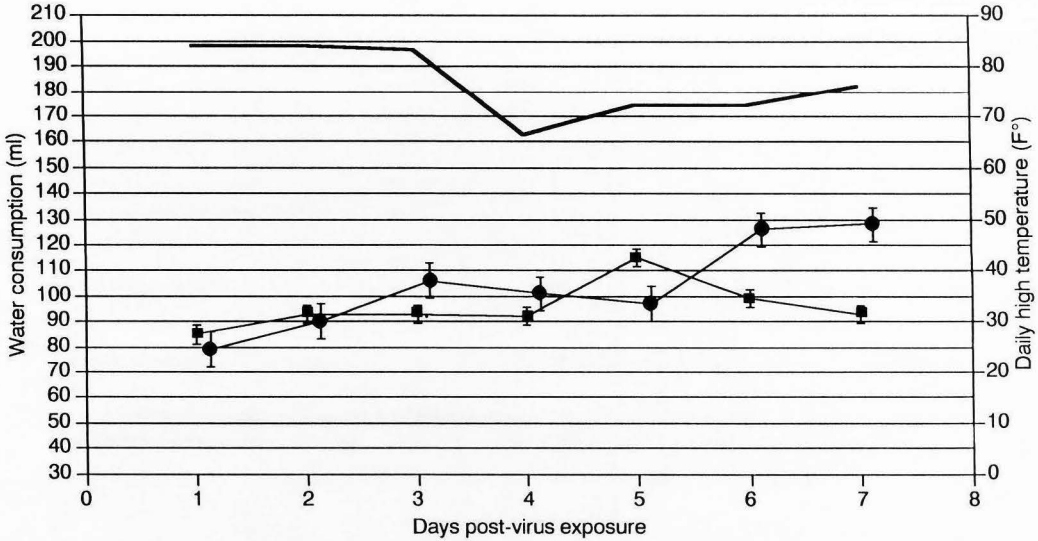

Fig. 7. Daily water consumption of uninfected control birds treated with ribavirin fog (the bold line indicates daily high temperature F°). —■— Virus Control —●— Normal Control —— Temp

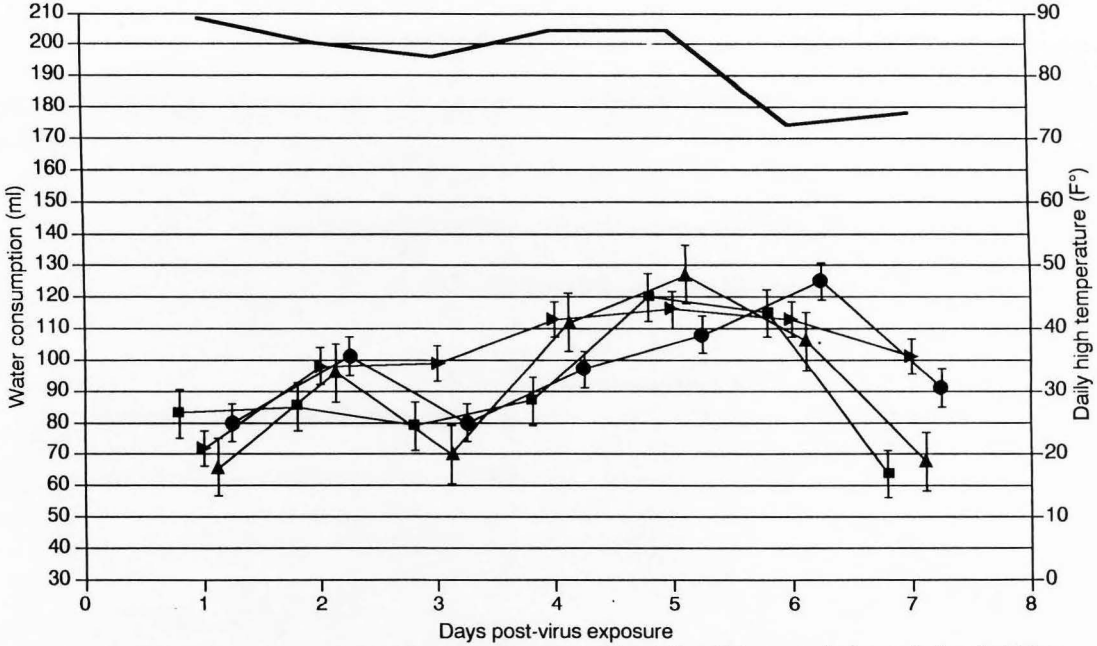

Fig. 8. Daily water consumption of infected poults treated with small-particle aerosol of amantadine (bold line indicates daily high temperature F°). —■— 30 min. —▶— 15 min. —▲— 7.5 min. —●— 3.8 min. —— Temp.

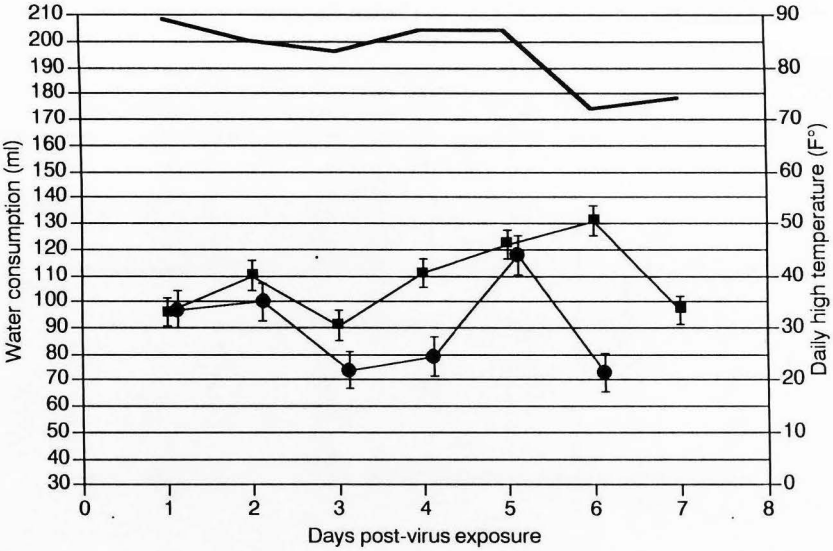

Fig. 9. Daily water consumption of uninfected control birds treated with small-particle aerosol of amantadine (bold line indicates daily high temperature F°). —■— Virus Control —●— Normal Control —— Temp.

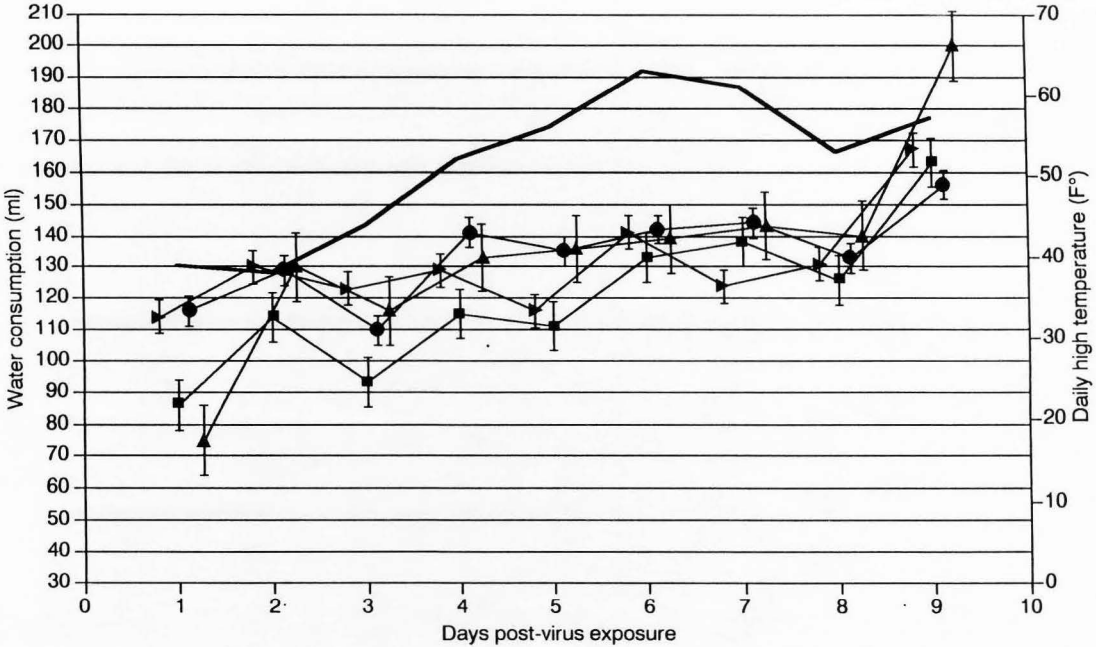

Fig. 10. Daily water consumption of infected poults treated with fog of amantadine (bold line indicates daily high temperature F°). —■— 3.0 sec —▶— 1.5 sec. —▲— .75 sec —●— .38 sec
—— Temp.

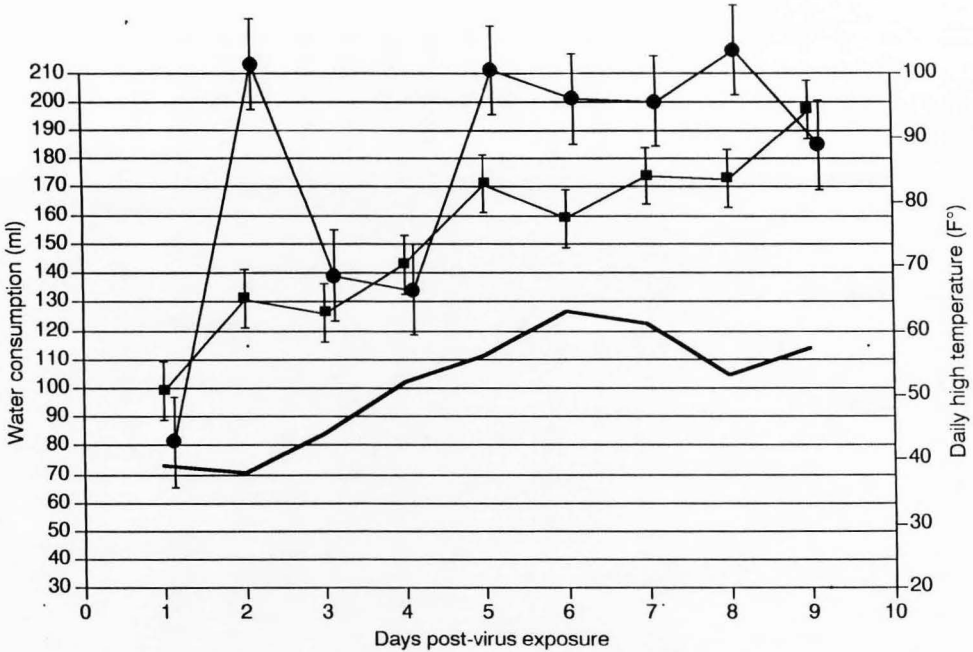

Fig. 11. Daily water consumption of uninfected control birds (bold line indicates daily high temperature F°). —■— Virus Control —●— Normal Control —— Temp

DISCUSSION

In Vitro Infectivity Potentiation

Passage of A/Turkey/Mass/65 in MDCK and MDBK cells resulted in a low and constant virus titer throughout, suggesting that the trypsinization of the virus did not potentiate its infectivity. This may be due to varying host passages, where a virus isolated from turkeys was passed in hens' eggs and then titered in the above cell lines resulting in a possible attenuation of cell line infectivity.

Another possible cause for the low titers may have been the improper exposure of the F protein at HA cleavage by the trypsin. This F protein is the active binding site after HA cleavage (Murphy and Webster 1985) .

A most plausible reason, however, is the fact that these virus pools were produced in hens' eggs, and consisted of pools of collected allantoic fluids. It is possible that the natural trypsin inhibitors ovomucoid and ovoinhibitor, found in hens eggs, inhibited the trypsin cleavage of the viral HA (Kassell 1970). Appleyard and Maber (1974) demonstrated that the presence of soybean trypsin inhibitor at the 0.5 mg/ml level greatly reduced the plaque-enhancing action of trypsin. This could create the condition seen in Table 3, where virus titers are constant throughout. The one exception in the trypsin experiment was the MDCK titration of A/Turkey/Mass in the presence of 40 ug/ml trypsin. At this point the virus titer jumped from 2.5 to 3.4, suggesting the possibility that the trypsin had overwhelmed the inhibitors and potentiated virus infection.

In Vitro Antiviral Studies

The antiviral activity of ribavirin and amantadine against AI was readily seen in vitro. Ribavirin's VR of >0.8 and TI of >11.6 were consistent with the strong inhibition of other strains of influenza reported by other researchers

(Huffman et al. 1973, Sidwell et al. 1972). The VR is greater than 0.8 due to the absence of toxicity in this test with ribavirin, which has been reported to demonstrate some toxicity at or above 320 μg/ml (Huffman et al. 1973; Sidwell et al. 1977).

The strong in vitro antiviral activity of amantadine against the AI viruses used in these studies (VR of 0.5 and TI >1.5) confirmed similar activity against other influenza viruses reported by Hoffmann (1973).

These in vitro studies run prior to the experiments in turkey poults indicated that the virus used in infecting the turkeys was indeed sensitive to the effects of both drugs.

Both amantadine and ribavirin would have had higher VR's if the virus had shown a greater CPE in the virus controls, making the test more definitive. The low VR's may have been due to the low virulent nature of the Utah virus isolates, and the possibly the low number of passages in cell culture achieved with these isolates.

In Vivo Infectivity Studies

The difficulty in obtaining definitive results in the antiviral portion of the project lies, in part, in the difficulty of producing quantifiable signs of illness with the viruses available to us. Demonstrable signs of illness were not seen using the first virus (A/Turkey/Mass/65) studied, other than mild sinusitis in 2 of 51 birds. This level of infection was considered unacceptable for use in antiviral studies. Attention then was turned to one of the fowl plague viruses, but this concept of using such a virulent agent was negated by the Utah State Veterinarian, because the risk of accidental release of a highly pathogenic organism was too great. Thus, the importation of a virus that would provide

highly quantifiable clinical signs in turkey poults could not be considered additionally.

Interest then was turned to 2 viruses isolated in turkey poults in Utah by Brigham Young University researchers. These viruses were reported to have caused in Utah turkey flocks mortality losses of 3-5% (Ahmed and Jensen 1987). Where *E. coli* infections complicated the virus disease, losses in flocks were reported as high as 20-30% (Ahmed and Jensen 1987). This Utah-isolated virus was then the best option for a strain that would be acceptable for use in the state.

These viruses offered, at best, mild signs of illness. This may have been at least in part due to the fact that the virus had been passed in hens' eggs at Brigham Young University and again at USU. This non-original host passage may have attenuated the virus for turkey poults. This idea is supported by data in Table 6, where lung scores are consistently higher in birds infected with virus passed once in turkey poults after the hens' egg passages. The difference, however, is minimized by the high lung score found in the normal controls.

This difficulty in producing quantifiable signs or lesions in experimentally infected birds has plagued other researchers as well (Beard 1984, Otsuki et al. 1981). This problem of low disease signs induced by AI, seems to be characteristic of the non-highly pathogenic strains of AI. Karunakaran (1984) found that of infected untreated birds, only 33% had recoverable virus in the lungs.

The unsuccessful efforts to assay recoverable virus left us unable to obtain consistent results. Turkey lung homogenates assayed in human embryonic lung cells (WI 38) showed no CPE at all, even in virus controls. Titrations utilizing primary CEF cells yielded erratic results. All samples were

titered on 2 separate occasions; in the first titration all samples (104 of 110) yielded positive CPE, including all the normal controls. The same pattern of low titers throughout the experiment, with occasional peaks in particular samples (except for normal controls) also has been reported by Karunakaran (1984) where 50% of all samples showed titers below 10^1.

Two weeks later, samples were rethawed and again titered. On this occasion 45 of 110 yielded positive CPE, and positives again were seen in the normal controls. If these titers actually were due to recovered virus, this reduction in titer levels easily would be explained by the freeze/thaw action on the virus. This left HA as the only reasonable quantifying measure of infection in this system. It is important to note the presence of positive HA in 3 out of 3 of the normal controls from the amantadine-fog experiment. This was of particular concern because positives were found throughout all 3 of the titration systems described above, indicating the possibility of cross contamination between infected birds and uninfected controls. Although every reasonable effort to eliminate this possibility was taken, it appears that it may have occurred.

In an effort to eliminate possible causes of these positive results, HI tests using antibody to Newcastle disease virus (Cytimmune, lot 84007, Lee Biomedical Research, San Diego, Ca.) did not demonstrate inhibition of HA in these samples. With the consistent nature of the HA in these samples (consistent in that it was still seen through 6 repeated freeze/thaw cycles) and their effect on the results of the 2 antiviral studies, concern remains as to what is the cause of this red blood cell reastion.

With this concern in mind, HA positive samples from the various normal controls were sent to the National Veterinary Services Laboratories (NVSL) with the approval of Dr. Stan Flora, Utah State Veterinarian. Dr. Dennis Senne

of the NVSL reported that, using primary embryonic kidney cells and embryonating chicken eggs, no evidence of viral infection was detected. HA tests on amnionic and allantoic fluids from embryos, where death was induced by these samples, were negative. It was thus determined by the NVSL that non-specific hemagglutination was occurring.

The use of health scores was found to be unproductive in this system due to the difficulty in visualizing signs of AI. The appearance of signs on different days post infection for different birds in a given treatment group diminished the usefulness of the average health score for the cage. This is due to the rapid appearance and abatement of what few signs were visible, leaving very few, if any, birds demonstrating signs concomitantly. Thus the health scores viewed were very low and demonstrated no significance.

In Vivo Antiviral Studies

Ribavirin treatment via small-particle aerosol caused significant reductions in lung HA titer and increases in water consumption, indicating reductions in the severity of the disease. This animal model system, utilizing strains of AI with low pathogenicity may not predict fairly the effectiveness of ribavirin's ability to prevent death of turkey poults, which would be likely with viruses of greater virulence. However, the data do suggest potential efficacy.

Positive results from ribavirin treatment of AI infection in chickens was demonstrated by Karunakaran (1984), although it was a mild response (approximately 15% reduction of recoverable virus) and treatment was via oral gavage.

The lack of significant reduction of any disease parameter in the ribavirin fog experiments indicates that treatment via large-particle aerosols are ineffective in prevention of treatment of AI in turkey poults. Presumably this

is due to the particle size of the aerosol, because other researchers have shown significant reduction in lung lesions, recoverable virus titers and significant increase in survival rate when using ribavirin in small-particle aerosols (Arensman, Dominik and Hilmas 1977). The function of particle size in efficacious treatment is dependent on the depth of the penetration of the drug-laden droplets into the respiratory tract. Large particles impinge on surfaces on the mucous membranes, nares and bronchi, while the smaller particles continue further into the interstitial spaces of the lung (Larson, Young and Walker 1976; Spendlove and Fannin 1979), where greater surface area facilitates more drug adsorption. However, there still remains the question of whether this occurs in the airsac or lungs of avian species.

Both the small-particle aerosol and the fog treatments utilizing amantadine produced significant reductions in the measured parameters. After considering the results of the small-particle aerosol results of the ribavirin experiments, it is surprising that the small-particle aerosol of amantadine only showed significant reduction of mean lung score. Aerosol (particle size is not known) work done by Karunakaran (1984) with amantadine utilizing 4-week-old birds (vs. 10-12 day old poults used in these experiments), showed 20% reduction of positives detected. Positive prophylactic effects of oral treatment with amantadine has also been demonstrated by Lang, Narayan and Rouse (1970). Because of the relative ease and availability of fog administration systems, the appearance of reduction in mean lung HA, although not significant, does suggests the need of further studies on this treatment system with amantadine.

The fog-administered treatment with amantadine indicated some promise, in that mean lung HA was significantly reduced in what appeared to be a dose-response fashion, although only the 2 longest treatment durations,

reflecting maximum amounts of amantadine therapy, caused significant reductions. This is consistent with the findings of other researchers using amantadine via oral gavage (Lang, Narayan and Rouse 1970), aerosol (Karunakaran 1984) and drinking water (Karunakaran 1984, Webster et al. 1985).

Water consumption data suggest a general decline in average consumption on day 3 post-virus exposure. This is similar to findings of McGahen and Hoffmann (1968), where at 36 hours post-infection they reported a reduction in water intake in mice. These researchers also found that the time after virus exposure when the reduced water intake occurred, varied with different virus strains, and used this to indicate the time to initiate treatment. Such data also suggests that treatment via drinking water likely would not be as effective as other treatment routes due to the fact that at the peak of infection the poults would be receiving less drug. However, Karunakaran (1984) found amantadine toxicity-induced death in drinking water treatments, suggesting adequate treatment levels may be reached.

Three of the 4 drug trials were conducted during the July and August months, raising the question of outside temperature effect on water consumption. However, the correlation coefficients between water consumption and daily high temperature suggest a minor effect of outside temperature on water consumption.

The results of these experiments suggest the possibility of successfully implementing a treatment program in the effort to control future outbreaks of AI. This statement is cautiously made, however, because of some important questions that remain unanswered. For example, the response of avian species infected with highly virulent strains of AI to treatment with ribavirin has

yet to be determined. Trials utilizing amantadine in this application were performed by Lang, Narayan and Rouse (1970).

Stronger demonstration of CPE and clinical signs would greatly improve the chances of making significant statistical decisions. Consistent recovery of virus would greatly enhance this system, and it seems appropriate to test for recoverable virus from multiple locations. Finally, better quantification of aerosol dosages would be required, perhaps even the detection of blood levels of the compounds used.

Overall, these experiments indicate the potential value of using aerosolized administrations of anti-influenza drugs for treating AI infections in domestic fowl. The method provides a means of dosing such birds to an approximately equal extent; of specifically targeting infected respiratory tissues, and of treating large numbers of birds the same time. When one considers the intensification procedures currently in use in poultry production, where literally thousands of birds may be in the same location, the applicability of such aerosolization techniques for controlling potentially devastating poultry losses due to AI appears quite obvious.

CONCLUSIONS

Avian Influenza is an economically important pathogen with losses in the last decade at approximately $100 million which justify looking for alternative control measures. The control of avian influenza is in its infancy, quarantine and depopulation yield questionable results, and better control measures seem necessary.

This turkey poult model using gross signs, lung HA and recoverable virus titers could be developed further for evaluation of antiviral agents in avian species. Use of health scores, however, was impractical due to their low and variable nature.

In this work, treatment of AI-infected poults with both small- and large-particle aerosols of amantadine and ribavirin suggested possible efficacy in the treatment of avian influenza in turkeys. However, the data derived in these experiments were not conclusive, and further investigation is warranted.

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