Suppression of Viral Pneumonia in Mice by a Microbial Product (APM)* (19739)

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For several years a systematic search for antiviral agents has been conducted in this laboratory. Two antibiotics were found which possessed antiviral properties, namely, Ehrlichin(1) and viscosin(2). Neither of these preparations appeared to be of sufficient interest to warrant further study and the search was resumed. Filtrates from cultures of various microorganisms recently isolated from soil were tested for antiviral activity against influenza A virus in both embryonated eggs and mice. Of the cultures tested, one, a culture of *Achromobacter sp.* 134,‡ produced a material endowed with a unique combination of biological characteristics. This material was found to suppress the development of pulmonary lesions in mice which resulted from infection with influenza A virus and to reduce the non-transmissible pneumonia induced by the intranasal instillation of large numbers of infectious particles of Newcastle disease virus. Further, attempts to demonstrate virucidal activity against these viruses were unsuccessful and antiviral activity in embryonated eggs was, at best, barely detectible by the usual methods.

Materials and methods. Pertinent information on the various viruses used in these studies and on the procedures followed in the use of embryonated eggs have been described (1,2). Hemagglutination tests were performed in the usual manner(3). Albino mice (Webster strain) weighing 18 to 20 g were infected intranasally under light ether anesthesia with 0.05 ml of appropriately diluted virus as indicated in the text. Parenteral injections of test materials were begun one hour or more after infection or as indicated. The lungs of each mouse were carefully examined and the degree of pulmonary consolidation was scored as described by Horsfall(4). The average weight of the lungs of the various groups of mice was frequently determined as an additional criterion.

Description of Achromobacter sp. No. 134. The bacteria measured approximately 0.7 by 2 to 10 μ and occurred singly or in short chains. The rods were gram-negative, non-acid-fast, non-spore-forming, and were motile by means of peritrichous flagella. The organism failed to grow anaerobically. On nutrient agar young colonies were circular, low convex, white, entire, adherent and measured 1.0 to 1.5 mm in diameter. Older colonies gradually developed radial striations with a lobate edge. A pellicle was usually formed in liquid media. Litmus milk was alkaline and reduced. An acid reaction was produced in media containing glucose, galactose and maltose and inorganic ($(NH_4)_2SO_4$) but not organic nitrogen. Nitrites were formed from nitrates. Production of H_2S was slight or absent. Indol was not formed. Starch and gelatin were hydrolyzed. Citrate was utilized as the sole source of carbon. The optimum temperature was 28°C to 36°C. The culture was isolated from soil.

Preparation of APM. Five ml amounts of an actively growing culture of *Achromobacter sp.* No. 134 were inoculated into each of a series of Blake bottles containing 250 ml of medium composed of 0.25% glucose, 1.0% peptone, 0.5% beef extract, and 0.1% yeast extract in distilled water. After incubation for 48 hours in the horizontal position the

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‡ This culture has been deposited in the culture collection of the Department of Microbiology under the number 134.

TABLE I. Suppressive Effect of APM on Pulmonary Lesions in Mice Infected with Influenza A Virus.

mg APM inj. SC on day:			Result–3rd day after infection*			
				Lesion score		Avg wt
0	1	2	I/T	L/M	%	lungs, g
0	0	0	21/22	48/105	46	
9	9	9	6/12	4/60	7	
3	3	3	12/12	8/60	13	
1	1	1	6/10	5/50	10	
.3	.3	.3	10/12	10/60	17	
.1	.1	.1	11/12	21/60	35	
			0/6 †			.18
0	0	0	15/15	53/75	71	.33
3	3	3	5/12	7/60	12	.22
0	3	3	12/12	20/60	33	.22
0	0	3	12/12	28/60	47	.24
0	3	0	11/12	19/60	32	.25
3	3	0	10/12	20/60	33	.23
3	0	0	8/12	14/60	23	.24

* Approximately 10000 ID_{50}.

† Uninfected controls.

SC = subcutaneously. I/T = No. of mice infected/total. L/M = Total lesion score/total max score. % = L ÷ M × 100.

Lesion score: ½ = <5% lung tissue consolidated; 1 = 5-25%; 2 = 26-50%; 3 = 51-75%; 4 = 76-100%; 5 = dead mouse with lungs consolidated.

culture was filtered through glass wool to remove the pellicle. The culture filtrates were pooled and acidified with concentrated HCl to pH 3.2 to 3.5. A whitish precipitate was formed which slowly flocculated. This precipitate was allowed to settle by gravity and the supernatant fluid was carefully removed by suction and discarded. The precipitate was then suspended in 10 to 20 volumes of distilled water and dissolved by the addition of 5 N NaOH to pH 8. The material was then reprecipitated 3 times in a similar manner. Before the final precipitation the solution was clarified using the Sharples supercentrifuge. The final (4th) neutralization was performed in a small volume of water which was dried from the frozen state. The average yield was approximately 110 mg of APM per liter of culture. This preparation was provisionally designated APM (acid precipitable material).

Experimental. Experiments with influenza A virus. Data from 2 typical experiments illustrating the suppressive effect of APM on the development of pneumonia in mice infected with influenza A virus are presented in Table I. Groups of 12 or more mice each were infected intranasally with approximately 10,000 ID_{50} of influenza A virus and injections of APM were begun one hour or more after infection as indicated. All mice were sacrificed on the 3rd day after infection and the degree of pulmonary consolidation and average weight of the lungs were recorded. The data indicate that (a) daily injections of APM were the most effective, (b) a delay of 24 hours in the time of treatment or a reduction in the number of injections of APM reduced its effectiveness, (c) a suppressive effect was still demonstrable when only one injection of APM was given 48 hours after infection, and (d) daily injection of as little as 0.3 mg of APM exerted a detectible suppressive effect on the development of pulmonary lesions. It is of interest in this connection to recall that the infective titer of lung tissue has been shown to be maximal 24 hours after infection with low dilutions of influenza A virus(5).

Comparative studies on the relative effectiveness of APM administered by various routes were carried out in similar experiments with influenza A virus. The data obtained indicated that intraperitoneal injection of APM was also effective but was toxic and that oral administration of 4 mg per day of APM was without effect. Intranasal instillation of APM-virus mixtures was also found to be ineffective in the following experiment. One ml amounts of serial decimal dilutions of influenza A virus were mixed *in vitro* with an equal volume of saline alone and saline containing 3 mg of APM per ml, respectively, and incubated for one hour at room temperature before inoculation into groups of 5 mice each by the intranasal route. The mice were observed for a period of 10 days and dead mice and mice still living on the 10th day after inoculation were examined and the degree of typical pulmonary consolidation was recorded. The end points (ID_{50}) of the 2 titrations were identical ($10^{-7.8}$) clearly indicating that APM was not virucidal for influenza A virus.

It was of interest to determine whether APM was more effective when mice were infected with a much smaller quantity of influenza A virus. In the experiment summarized in Table II, groups of 20 mice each were

TABLE II. Effect of APM on Degree of Pulmonary Consolidation in Mice Infected with Sublethal Dose of Influenza A Virus.

APM	Result–10th day after infection* Lesion score I/T	L/M	%
Control	17/20	34/100	34
1 mg SC × 10	5/20	9/100	9
3 mg SC × 10	6/19	6/95	6

* 10 ID_{50}.

TABLE III. Effect of APM on Rate of Death of Mice Infected with 10000 ID_{50} of Influenza A Virus.

APM: dose/day	I/T	Cumulative % mortality on day: 2	3	4	5	6	7
Control	19/20	0	15	45	80	85	95
1 mg SC	20/20	0	0	20	50	95	95
3 "	20/20	0	0	0	25	60	95

TABLE IV. Effect of Culture Filtrate and APM on Influenza A Virus in Eggs.

Type of test	Test material	Result I/T	Result Avg hemagglutinin titer
Contact	Control	10/11	626*
	Culture filtrate	9/11	380
Therapeutic	Control	13/14	731
	Culture filtrate	11/16	270
Protection	Control	14/15	955
	Culture filtrate	12/14	340
Contact	Control	7/7	1473
	2.5 mg APM†	4/4	840
	1.25	7/8	740

* Reciprocal of avg hemagglutinin titer of allantoic fluid collected 48 hr after infection with 10-100 ID_{50}.

† Sterilized with ethylene oxide.

Contact test = test material mixed *in vitro* with virus and incubated 1 hr at room temperature before inoculation.

Therapeutic test = test material inj. 1 hr after infection.

Protection test = test material inj. 1 hr before infection.

infected with a sublethal dose (10 ID_{50}) of virus and were injected daily subcutaneously with one and 3 mg of APM, respectively, beginning one hour after infection. Twenty similarly infected but untreated mice served as the control group. All mice were sacrificed on the 10th day after infection and the degree of pulmonary consolidation was noted. The data indicate that daily injections of APM definitely suppressed the development of pulmonary lesions in mice infected with a sublethal dose of influenza A virus. However, it would appear that the effectiveness of APM in suppressing the development of pneumonia was not markedly increased when the size of the infecting dose of virus was greatly (1,000x) reduced (Table I).

It was of obvious importance to study the effect of APM on the rate of death of mice infected with a lethal dose of influenza A virus. The results of a typical experiment are summarized in Table III. This experiment was identical with the preceding one except that a lethal dose of virus (10,000 ID_{50}) was employed. The data show that daily injections of 3 mg of APM delayed death of the mice by approximately 2 days and that when the daily dose of APM injected was decreased to 1 mg the delay in death was reduced.

An attempt to demonstrate a reduction in the infective titer of pools of 8 lungs each collected 24 hours after infection was unsuccessful. The mice were injected subcutaneously with 5 mg of APM one hour before intranasal inoculation of 10 and 100 ID_{50} of influenza A virus, respectively.

Considerable time and effort were spent in attempting to demonstrate antiviral activity against influenza A virus in embryonated eggs using both culture filtrate and APM. Suppressive effects, though regularly observed, were minimal. The various preparations were rendered suitable for injection into eggs by the addition of penicillin (500 units per ml) and neomycin (200 units per ml). Control inocula were similarly treated. The results of several typical experiments are summarized in Table IV. The data indicate that culture filtrate known to be effective in suppressing the development of pulmonary lesions in mice was, at best, capable of effecting a slight (approximately 2-fold) reduction in the average hemagglutinin titer of allantoic fluid collected 48 hours after infection with 10-100 ID_{50} of influenza A virus. However, this slight reduction in the formation of viral hemagglutinin was obtained whether the culture filtrate was mixed *in vitro* with the virus or was injected one hour before or one hour after infection. It was hoped that APM would prove to be more effective than culture filtrate. However, this was not the case. A solution of APM

containing 5 mg per ml was sterilized with ethylene oxide(6), and was tested for antiviral activity by means of the contact test. As with culture filtrate a slight (approximately 2-fold) reduction in viral hemagglutinin was obtained. Attempts to demonstrate a reduction in the infective titer of allantoic fluid collected 24 hours after inoculation were unsuccessful. The eggs received potent culture filtrate one hour after infection with 10 ID_{50} of virus. It is clear that the embryonated egg was not a suitable host for the demonstration or detection of antiviral activity of APM against influenza A virus when the usual methods were employed.

APM and culture filtrate both failed to inhibit hemagglutination *in vitro* by influenza A or B viruses. Infected allantoic fluids were titrated in the usual manner(3) in the presence of culture filtrate, APM (2 mg per ml), broth and saline, respectively. The hemagglutinin-test material mixtures were incubated for one hour at 36°C before the addition of chicken erythrocytes. Identical end points were obtained in each of the various titrations with influenza A and B viruses, respectively.

Experiments with Newcastle disease virus (NDV). Ginsberg(7) has shown that the non-transmissible pneumonia in mice which follows intranasal inoculation of NDV paralleled the reactions between virus and host cell save that formation of new infectious particles did not occur. Further, when the maximum of pneumonia was observed there remained less than 0.1% of the original NDV inoculum and "immature or incomplete virus"(8-11) could not be demonstrated in lung tissues by complement fixation or hemagglutination. This unique host-virus relationship was found to be affected by APM in the experiments described below.

A large number of mice were inoculated intranasally with 0.1 ml of undiluted allantoic fluid containing 10^9 infectious units of NDV. Beginning one hour after inoculation and daily thereafter groups of 15 mice each were injected subcutaneously with various amounts of APM as indicated in Table V. Inoculated but otherwise untreated mice served as controls. All mice were sacrificed on the 3rd day after inoculation (at the time of maximum pneumonia(7)) and the degree of pulmonary consolidation and the average weight of the lungs were recorded. It is clear from the data that daily injection of as little as 0.03 mg of APM suppressed but did not prevent the development of pneumonia.

TABLE V. Effect of APM on Development of Pneumonia Induced by Newcastle Disease Virus in Mice.

APM	Result—3rd day after inoc.* I/T	Lesion score L/M	%	Avg wt lungs, g
	0/4†			.18
Control	30/30	100/150	67	.27
1 mg SC × 3	12/15	20/75	27	.21
.3	13/15	20/75	27	.22
.1	14/15	19/75	25	.22
.03	10/15	18/75	24	.23
.01	12/15	34/75	45	.25

* 10^9 ID_{50}.
† Uninoculated controls.

No evidence of inactivation of NDV *in vitro* could be demonstrated when the virus was titrated in the presence of saline, culture filtrate, or APM, respectively, before inoculation into embryonated eggs.

Properties of APM. Preliminary qualitative data on the properties of APM were obtained in the following manner. Preparations of APM were treated as described below and tested for potency in mice previously inoculated intranasally with 10,000 ID_{50} of influenza A virus or 10^9 infectious units of NDV. One mg amounts of the various preparations were injected daily subcutaneously into groups of 12 mice each beginning one hour after instillation of virus. All mice were sacrificed on the 3rd day after infection and the degree of pulmonary consolidation and average weight of the lungs was determined (as exemplified in Tables I and V). The following results were obtained. The potency of aqueous solutions of APM was not completely destroyed by (a) exposure to a temperature of 120°C for 45 minutes at pH 9; (b) dialysis against running tap water for 24 hours; or (c) serial extraction of the same solution of APM with n-butanol, anesthetic ether, chloroform, petroleum ether, ethyl acetate and benzene. Half saturation of an aqueous solution of APM with ammonium sulfate resulted in the immediate formation of a flocculent precipitate which was

found to be both acid precipitable and potent.

No evidence of antibacterial or antifungal activity could be demonstrated when the streak-dilution method(12) was employed. A concentration of 5 mg of APM per ml of agar did not inhibit the growth of *Escherichia coli, Micrococcus pyogenes* var. *aureus, Pseudomonas aeruginosa, Shigella sonnei, Bacillus cereus, B. subtilis, Mycobacterium tuberculosis* (strain 607), *Streptomyces griseus, S. fradiae, Candida albicans, Aspergillus niger* or *Penicillium notatum.*§

Discussion. The most interesting characteristic of APM is its capacity to suppress the development of non-transmissible pneumonia in mice induced by the intranasal instillation of large numbers of infectious particles of NDV. Ginsberg(7) has suggested that the pneumonia following inoculation of NDV may be the result of the injurious action of a large amount of virus. However, he points out that the possibility of multiplication of non-infectious virus was not definitely eliminated. APM might affect either or both of these factors. On the other hand, it is equally plausible to visualize the action of APM as primarily affecting the lesion itself. It is clear that the *continued* presence of large numbers of infectious particles of NDV in the lung is not essential for the development of pneumonia(7). However, it is not known whether the development of the lesion is dependent on the continued presence of intracellular NDV in one form or another.

Attention should be drawn to the studies of Horsfall and Ginsberg with pneumonia virus of mice(13) and influenza A virus(5) as well as with NDV(7) which indicated that pulmonary lesions are formed at a rate different from that of infective virus. These investigators suggested that factors other than viral multiplication *per se* are involved in the production of lesions. The fact that APM suppresses viral pneumonia in mice in the presence or absence of the formation of new infectious virus is in agreement with this suggestion.

Studies on the mode of action of APM, its antiviral spectrum, and its chemical and physical properties are in progress.

Summary. A material (APM) produced by a culture of *Achromobacter sp.*, was found to possess a unique combination of biological characteristics. (a) APM was not inhibitory to representative species of bacteria or fungi *in vitro*. (b) Preparations capable of suppressing the development of pulmonary lesions in mice previously infected with influenza A virus were found to be devoid of virucidal activity *in vitro* and failed to affect the production of infectious virus in lung tissue or allantoic fluid. (c) The mouse was a far better host than the embryonated egg for the demonstration or detection of inhibitory effects of APM on influenza A virus. (d) Hemagglutination *in vitro* by influenza A or B virus was not affected by the presence of APM. (e) The most interesting characteristic of APM was its capacity to suppress the development of non-transmissible pneumonia in mice induced by intranasal inoculation of Newcastle disease virus.

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