Induction of Viral Interference in Mice by Aerosols of Inactivated Influenza Virus.* (28387)

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With one exception(1), observations of interference with influenza virus multiplication in the intact animal have been limited to the double infection type of interference, in which one multiplying virus inhibits the multiplication or pathogenic effects of another. However, influenza viruses may be so inactivated by heat or ultraviolet irradiation, that their capacity to multiply is destroyed, but their ability to interfere with multiplication of infective virus is preserved(2,3,4).

It is now clear that interference is a localized intracellular phenomenon, and that those cells in direct contact with interfering virus are affected(5). Influenza virus infection is usually restricted to cells lining the respiratory tract. Hence, the intranasal or aerosol administration of partially inactivated preparations of virus might be expected to induce an altered state of local resistance and thus affect the course of infection upon subsequent challenge with fully virulent homologous or heterologous virus. The present studies were designed to investigate this possibility.

Materials and methods. Mice. CFW male mice averaging 20 g in weight were employed in all experiments. Animals were housed in glass jars in groups of 5 or in stainless steel containers in groups of 10.

Lungs were removed aseptically at designated intervals and ground in glass tubes with teflon grinders. Ten per cent suspensions in 0.01 M phosphate buffered 0.85% saline, pH 7.1 (PBS), (penicillin 500 U/ml; streptomycin 5 mg/ml) were centrifuged at 3000 RPM at 4°C for 15 minutes. Supernatant fluids were diluted in PBS in 10-fold increments. The viral content was titrated immediately in eggs or the undiluted lung suspensions were frozen in plastic tubes in a solid CO_2-ethanol mixture and stored at −65°C for later titration.

Eggs. 10 day old White Leghorn chick embryos incubated at 38°C were employed in the titration of lung suspensions.

Viruses. The CAM strain of influenza A1 virus was employed in most experiments as the inactivated, interfering virus.

The Lee strain of influenza B virus was employed in most experiments as the challenge virus. In some experiments a strain of Asian influenza virus, A2/Cornell/221/57 was used as the challenge virus.

Viral titrations. Virus content of ground suspensions of individual mouse lungs was measured by infectivity titrations in eggs, using 3 eggs per dilution. Allantoic fluids were harvested after 40-48 hours of incubation and tested for viral hemagglutinin in 1:4 dilution with 1% human "O" RBC. Fifty per cent titration end points were calculated according to the method of Reed and Muench.

Scoring of pulmonary lesions. A modification of the maximum score method(6) was used in which the extent of pulmonary lesions was expressed as a percentage of total lung surface.

Inactivation of interfering virus. Chick embryos were inoculated with 10^5-10^6 EID/50 and the allantoic fluid harvested 24 hours later. Sterile fluids were pooled and centrifuged at 8000 RPM for 5 minutes. Supernatant fluids were dialyzed for 18 hours against phosphate buffered saline (PBS) at 4°C at a ratio of 20:1. The fluid in 15 ml aliquots then was exposed to ultraviolet light in a petri dish for 45 seconds with continuous mixing with a magnetic stirrer. The light source was a GE 7½ watt ultraviolet lamp mounted 7 inches above the dish. The virus then was concentrated in the ultracentrifuge

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(Spinco). Inactivated virus suspensions were sedimented at 44,300 *g* for 3 hours. The supernatant fluid was removed by decanting and the pellet resuspended in 1.0 ml of PBS by gentle refluxing with a capillary pipette. The resuspended pellet was diluted in PBS to a final volume 1/10 the starting volume. Hemagglutination (HA) titer, interfering activity *in ovo*, and residual infectivity in eggs were tested. Viral suspensions prepared in this manner had no demonstrable infectivity in eggs except in the presence of cortisone.

In other experiments several modifications of these basic procedures were made. The dialysis procedure was omitted, and the unirradiated virus was concentrated 100-fold in the ultracentrifuge and then irradiated. When these highly concentrated preparations were tested, it was found that to destroy residual infectivity, longer periods of irradiation, and on some occasions, incubation at 37°C for 24 hours were necessary.

Intranasal inoculation. Mice were lightly anesthetized with ether and .04 ml of inactivated virus or saline was instilled intranasally.

Aerosol procedure. In most experiments, the inactivated and the challenge viruses were both delivered as aerosols in a closed chamber in which the mice were exposed for appropriate intervals. The conditions under which the aerosol procedure was carried out are as follows: A continuous vacuum was applied so that 25 liters of air/min flowed through the chamber. Compressed air flowed through a number 40 *Vaponephrin* nebulizer under 15 lb pressure/square inch at a rate of 5 liters/min. The remaining air entering the chamber (20 liters/min) was room air. Under these conditions, 0.3 cc of nebulizer fluid/min was introduced into the chamber as an aerosol mist. Mice were placed in a wire cage inside the chamber for a period of 30 minutes to 3 hours.

In preliminary studies, the uniformity of pulmonary virus titers resulting from intranasal and aerosol-induced infection was assessed. The mean standard deviation of the 72 hour virus titers of individual mouse lungs was 0.4 Log_{10} EID/50 for aerosol-induced infections, and 0.8 Log_{10} EID/50 for intranasal infections.

Results. In Table I are presented the results of 2 experiments in which influenza B (Lee) and influenza A1 (CAM) were administered reciprocally by aerosol as irradiated (interfering) or challenge (infective) viruses. The irradiated Lee virus had an HA titer of 1:1024 and the irradiated CAM virus an HA titer of 1:13,280. Mice were exposed for 3 hours to the aerosols of noninfective irradiated virus 24 hours prior to the aerosol administration of heterologous, infective virus (estimated 100 MID/50). Lungs were harvested at the indicated intervals 2-8 days after challenge and the virus content titrated in eggs. The mean pulmonary viral titer of mice previously exposed to inactivated virus was significantly lower 2-6 days after challenge. The extent

TABLE I. Reduction of Pulmonary Virus and Lesions as Assessed by Study of Reciprocal Pairs of Irradiated and Challenge Influenza Viruses.

Aerosol inoculation			Pulmonary virus titer*		Lesion score (%)	
Non-infective irradiated virus (day 1)	Challenge virus (day 0)	Day	U-V virus group	Saline group	U-V virus group	Saline group
B (Lee)†	A1 (CAM) (100 MID/50)	2	4.1	5.2		
		4	5.3	6.7		
		6	3.3	5.9	18	43
		8	3.4	3.5	38	92
A1 (CAM)‡	B (Lee) (100 MID/50)	2	2.3	3.4		
		3	2.4	4.2		
		4	2.9	3.8		
		5	1.4	2.8		
		7	—	—	0	20

* Log_{10} (EID/50). Mean of titers obtained by titration of lungs of 5 animals.
† Hemagglutination titer 1:1,024; *in ovo* interference titer 1:4.
‡ Hemagglutination titer 1:13,280; *in ovo* interference titer 1:32.

TABLE II. Duration of Interfering Effect with Influenza A2 Virus Challenge Following Administration of Irradiated Influenza A1 Virus* by Aerosol.

Interval between irradiated and challenge viruses	3rd day pulmonary virus titer†		
	U-V virus pretreatment		Saline pretreatment
6 hr	5.4		5.3
24 "	3.5	P<.05	5.2
72 "	3.6	P<.05	5.4
5 days	3.8	P>.05	4.3
7 "	3.0	P>.05	4.0

* Irradiated influenza A1 (CAM) by aerosol (Hemagglutination titer 1:13,280).

† Mean pulmonary virus titer (EID/50 $\log_{10}$) 3 days after challenge virus; 5 animals in each group individually titrated. The challenge virus was mouse-adapted influenza A2 virus.

of pulmonary lesions observed 6, 7, or 8 days after infection was similarly considerably less in animals that had been exposed to irradiated virus. Thus, reduction in peak viral titers early in the course of influenza virus infection was associated with reduction in gross pulmonary lesions in the later stages of infection.

Duration of effect of inactivated virus. Twenty-five mice were exposed for one hour to the preparation of irradiated CAM virus used in the experiment previously described. Control animals were exposed to saline. Six hours and one, 3, 5 and 7 days after this treatment, 5 animals in each group were challenged by exposure to an aerosol providing an estimated 100 MID/50 of A2 influenza virus (A2/Cornell/221/57) not adapted to the mouse. Three days after each challenge exposure, animals were autopsied and viral titers of individual lungs were determined in eggs. The results are summarized in Table II. With an interval of 6 hours between exposure to irradiated virus and challenge virus no protection was evident; *i.e.*, the mean 3-day pulmonary virus titers were identical in control and experimental animals. When the interval was one or 3 days, significant reductions in viral titers occurred (P less than .05 at each interval). With 5 and 7 day intervals between pretreatment and challenge, the reductions in 3-day viral titers were not statistically significant.

Effect of interference on subsequent level of immunity. It was of interest to determine whether infection modified by inactivated virus would result in a diminished immune response to the virus employed in the challenge infection.

Fifteen mice were pretreated by exposure to an aerosol of concentrated, irradiated A1 (CAM) virus. The hemagglutination titer of this preparation was 1:10,280 and interfering activity in eggs could be demonstrated at a dilution of 1:64. Fifteen control animals were exposed to a saline aerosol. The following day 10 of the 15 animals in each group were challenged with an estimated 100 MID/50 of influenza A2 virus delivered by aerosol. Pulmonary virus was individually titrated in 5 infected animals from each group 72 hours after infection (Table III). Virus titers were significantly lower (P less than .05) in the animals pretreated with irradiated virus (Group 6), than in control mice (Group 3). Ten days after the first challenge, the 5 previously infected animals in each group were exposed to a second challenge identical to the first. The 5 control (previously uninfected) animals in each

TABLE III. Development of Specific Immunity to Re-challenge in Mice Previously Treated with Heterologous Irradiated Virus.

Group	Day −1	Challenge, day 0	Lung titer,* day 3	Challenge, day 10	Lung titer,* day 13
1	Saline	Saline	—	A2	5.0
2	"	A2	—	A2	<1
3	"	A2	5.2	—	—
4	u-v A1‡	Saline	—	A2	4.4
5	"	A2	—	A2	<1.9†
6	"	A2	3.5	—	—

* $\log_{10}$ (EID/50).

† <1 in 3/5 animals; 2.7 and 3.7 in the other 2 mice.

‡ Exposure for 3 hr to aerosol of irradiated CAM virus. Hemagglutination titer 1:10,280; interference *in ovo* at a dilution of 1:64.

TABLE IV. Correlation of Interfering Activity of Inactivated Influenza Virus in Mice with Other Virological Activity.

		HA* activity	Enzyme activity	*In ovo* interference	Interference in mice
1	Irradiated CAM virus by aerosol	+	+	+	+
2	Supernatant intranasally	±	0	±	0
3	Irradiated and heated virus	+	?	0	0
4	Receptor destroying enzyme (RDE)	0	+	0	0
5	"Interferon"†	0	0	0	0
6	Irradiated CAM virus, intraperitoneally	+	+	+	0

* Hemagglutinating.
† Lung suspension from mice obtained 24 hr after exposure to irradiated CAM virus.

group also were exposed to the same aerosol mist of A2 virus. Three days later pulmonary virus content of all 20 animals was determined. Following a second challenge with A2 virus, no pulmonary virus was demonstrable in the mice not given irradiated virus before the first challenge (Group 2). In contrast, pulmonary virus was demonstrable in 2 of the 5 mice in which the initial infection had been preceded by administration of irradiated heterologous virus (Group 5). These titers were lower than those of non-immune animals exposed to the same challenge (Groups 1 and 4). Thus, modification of infection by heterologous inactivated virus was followed by reduced immunity to the challenge virus in the post-infection period.

Mechanism by which irradiated influenza viruses modify the course of infection with heterologous virus in mice. Table IV summarizes schematically, experiments designed to study the mechanism by which irradiated virus acts in the mouse to alter the course of infection with heterologous, fully infective virus. Partially inactivated virus, which was capable upon aerosol administration of reducing viral titers upon subsequent challenge with A2 virus, had no effect when given intraperitoneally. The modification of infection induced by aerosol administration of irradiated virus therefore was dependent on route of administration.

When an irradiated virus preparation was further inactivated by heating at 37° for 48 hours, it maintained most of its hemagglutinating activity, but lost its ability to induce interference *in ovo*. This preparation given intranasally had no effect in reducing 72 hour virus titers in mice challenged with A2 virus 24 hours after pretreatment.

Supernatant fluid of the CAM virus preparation after centrifugation had no effect when given to mice 24 hours before challenge with A2 virus. The supernatant fluid had a hemagglutination titer of 1:16, but had only minimal *in ovo* interfering effect. Thus the loss or absence of interfering activity *in ovo* correlated with the absence of interfering activity in mice.

Efforts were made to determine whether the viral interference induced in mice by irradiated virus was related to receptor destroying activity. The enzymatic activities of an irradiated CAM virus preparation and of non-viral receptor destroying enzyme (RDE) were assayed by the destruction of mouse lung hemagglutinating inhibitor. RDE was diluted to equal the enzymatic activity of the irradiated virus and was administered to mice intranasally. Upon challenge 24 hours later with Lee virus, no protection was evident.

Efforts were also made to demonstrate an interferon-like substance in mouse lungs. Ground suspensions were prepared from lungs of mice given irradiated CAM virus intranasally 24 hours previously. When these suspensions were inoculated intranasally into mice, and the animals challenged 24 hours later with Lee virus, there was no reduction in 72 hour pulmonary virus titers.

Discussion. The modification of influenza virus infection in mice observed in these experiments was probably the result of classical interference induced by heterologous inactivated virus. Such interference is localized to cells *directly* exposed to inactivated virus(6). In these experiments, reduced viral titers were observed only when inactivated and challenge viruses were administered by the same route, an observation in keeping with

the purely local nature of interference.

The demonstration in these experiments that antigenically unrelated strains of influenza virus were capable of reducing the multiplication of one another not only eliminates the possible effect of specific immunity but suggests another similarity to interference in cell cultures(7).

The time interval required between the administration of inactivated and challenge virus suggests the possibility that the interference induced in these experiments was mediated by interferon. When chick embryos are inoculated with influenza virus, the peak titers of interferon in the allantoic fluid are not attained immediately(8,9). Isaacs and Hitchcock have presented evidence that mice produce interferon during the course of influenza virus infection, and that peak titers of interferon are attained shortly after peak pulmonary virus titers are reached. An attempt in these experiments was made to isolate an interferon-like substance from the lungs of mice given inactivated virus intranasally 24 hours previously. The fact that no interferon-like material was found may be explained by the insensitivity of the test system employed (*i.e.*, the lungs of living mice). On the other hand, all interference may not be mediated by interferon. Schlesinger(11) has pointed out that partial or abnormal cycles of multiplication may be expected to occur when favored by the presence of functionally deficient particles of a heterologous virus. Another possible mechanism is that interfering and challenge viruses may compete intracellularly at the site of essential reactions for virus replication(12).

The effect of interference on the induction of specific immunity following challenge infection was of interest. The reduction of virus multiplication induced by interference was sufficient to diminish the level of immunity that developed to the challenge virus, but sufficient virus multiplication took place to produce significant levels of immunity. This result is in accord with earlier studies of simultaneous infection of hamsters with influenza A and B viruses in which lessened antibody response to both viruses followed (13).

Summary. Inactivated influenza viruses administered to mice intranasally or by aerosol induced transient heterologous immunity to infective influenza viruses. This immunity was reflected by reduction of pulmonary viral titers and by a reduced occurrence of gross lesions. This effect is considered to be an example of viral interference in that it required time to develop and persisted for at least 3 days; and because protective activity of inactivated viruses in mice could be correlated with interfering activity in the chick embryo. Interference modified but did not completely inhibit infection, and strain specific immunity of lessened degree developed to the challenge virus following the modified infection.

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