



Title	The Nature of The Immunity Evoked by Infection of Mice with Avirulent Influenza Virus
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Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1972, 15(1), p. 31-37
Version Type	VoR
URL	<a href="https://doi.org/10.18910/82729">https://doi.org/10.18910/82729</a>
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# THE NATURE OF THE IMMUNITY EVOKED BY INFECTION OF MICE WITH AVIRULENT INFLUENZA VIRUS

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(Received October 16, 1971)

**S**UMMARY Cross-protection among influenza viruses was studied in mice. Mortality, consolidation of the lungs and neutralizing antibody were used as indices of protection. Immunization of mice with live avirulent A / Okuda / 57 (H<sub>2</sub>N<sub>2</sub>) or A / Osaka / 9 / 70 (H<sub>3</sub>N<sub>2</sub>) had a protective effect against challenge with virulent A / FM1 / 47 (H<sub>1</sub>N<sub>1</sub>) while immunization with live avirulent B / Osaka / 3 / 71 resulted in no protection against A / FM1 / 47 (H<sub>1</sub>N<sub>1</sub>). No neutralizing antibody or hemagglutination inhibition antibody against A / FM1 / 47 (H<sub>1</sub>N<sub>1</sub>) was found in the sera or lung exudates of mice immunized with live A / Okuda / 57 (H<sub>2</sub>N<sub>2</sub>), even when mice were immunized 4-5 times at weekly intervals. Moreover, pooled and concentrated sera or lung exudates of mice immunized with A / Okuda / 57 (H<sub>2</sub>N<sub>2</sub>) showed no neutralizing activity against A / FM1 / 47 (H<sub>1</sub>N<sub>1</sub>). The possible role of immunoglobulin in lung secretions or sera is discussed.

## INTRODUCTION

For more than a decade, live attenuated influenza vaccines have been tested in field trials (Stuart-Harris, 1970; Okuno and Nakamura, 1966). But there are still many problems to be solved about these vaccines, such as lack of laboratory markers for attenuation of virus and evidence for the overwhelming advantage of using live vaccine (Stuart-Harris, 1970). In the previous report (Kurimura et al., 1971), we described some characteristics of infection immunity against influenza in mice. Table 1 summarizes previous experiments. Preim-

munization with live A / Okuda / 57 (H<sub>2</sub>N<sub>2</sub>) effectively protected mice against challenge with the heterotypic virus, A / FM1/47 (H<sub>1</sub>N<sub>1</sub>). On the other hand UV-killed A / Okuda/57 (H<sub>2</sub>N<sub>2</sub>) virus was ineffective. The broadened spectrum of immunity evoked by immunization with a live avirulent influenza virus strain must be evidence for the advantage of using live influenza vaccine. Recently, Waldman et al. (1970) reported that the characteristics of secretory IgA were less specific than those of serum IgG. If this is so, the route of immu-

nization must be very important in influenza vaccination. In this paper the characteristics of immunity evoked by infection of mice with a live avirulent influenza virus are discussed.

## MATERIALS AND METHODS

### 1. Mice

Four or 5 week old inbred female mice of the ddO strain were used throughout. At the beginning and end of experiments, control mice were checked for HVJ infection.

### 2. Influenza virus

As avirulent strains in mice, A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>), A/Osaka / 9 / 70 (H<sub>3</sub>N<sub>2</sub>) (Hong Kong type) and B/Osaka/3/71 were used. The latter two strains were freshly isolated in our laboratory. A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>) is known to have similar laboratory characteristics to another attenuated virus (Willers and Beare, 1970). The A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) strain was used as the virulent strain for challenge. Ten day old fertile eggs were infected with diluted virus samples and two (or three, in the case of type B virus) days later the allantoic fluid was harvested. Its virus titer was determined at the time of infection of mice by the EID<sub>50</sub> test or hemagglutination of chick red blood cells.

### 3. Immunization and challenge of mice

For challenge of mice with the virulent strain A/FM1/47(H<sub>1</sub>N<sub>1</sub>), the inhalation method using a nebulizer was adopted (Nakamura, 1965). Mice were immunized by inhalation, intraperitoneal injection or subcutaneous injection of virus. The interval between immunization and challenge was 14 days. Mice were observed for two weeks after infection and at the end of the experiments surviving mice were sacrificed by exsanguination and their lungs were checked carefully for consolidation. The extent of consolidation is expressed as 0, 1, 2, 3 or 4, representing 0, 25, 50, 75 or 100% consolidation of the lungs. To obtain lung exudates, the lungs were taken out together with the trachea and each lung was washed with 0.5 ml of phosphate buffered saline (PBS). Sera of mice were used for assay of NT and HI antibody and lung exudates for assay of NT antibody.

### 4. Neutralization test and hemagglutination inhibition test

These tests were done as described elsewhere (Kurimura et al., 1971). The HI antibody titer is expressed as the log<sub>2</sub> of the reciprocal of the highest serum dilution causing inhibition of hemagglutination. The NT antibody titer is expressed as the reciprocal of the highest dilution of serum showing neutralization of virus in more than 50% of the eggs. Pooled sera or lung exudates, were concentrated by ultrafiltration in Visking tubing (size 8/32) under reduced pressure. After concentration, sera or lung exudates were dialyzed against 50 volumes of Eagle's MEM for several hours at 4 C.

## RESULTS

As described in the previous paper, immunization of mice with live avirulent virus 9, 12 or 22 days before challenge with heterotypic virulent virus, resulted in protection against virulent virus (Table 1).

These experiments showed that the mice developed broadened immunity after immunization with live type A influenza virus. The experiments shown in Tables 2 and 3 were performed to examine the spectrum of immunity evoked by immunization with various

TABLE 1. Summary of previous experiments<sup>a</sup>

Immunization <sup>b</sup>	Challenge <sup>b</sup>	Interval between immunization and challenge (days)	Effectiveness of immunization
live A/Okuda/57 (H <sub>2</sub> N <sub>2</sub> )	A/FM1/47 (H <sub>1</sub> N <sub>1</sub> )	9	+
		12	+
		22	+
UV-killed A/Okuda/57 (H <sub>2</sub> N <sub>2</sub> )	A/FM1/47 (H <sub>1</sub> N <sub>1</sub> )	12	—

<sup>a</sup> Summary of experiments described in the previous paper (Kurimura et al., 1971).

<sup>b</sup> Immunization and challenge were done by the inhalation method.

types of influenza virus. Mice were immunized by the inhalation method with A/Osaka/9/70(H<sub>3</sub>N<sub>2</sub>) (Hong Kong type) and 14 days later they were challenged by A/FM1/47(H<sub>1</sub>N<sub>1</sub>) (Table 2). Judging from the death rate and incidence of consolidation of the lung, these heterotypic strains gave cross protection and immunized mice were more resistant to chal-

lenge virus than nonimmunized controls. These data suggest that cross protection after immunization with live virus is a common phenomenon in various strains of type A influenza virus. But this type of cross immunity does not give a sufficiently broad immunity to overcome the antigenic heterogeneity between A (H<sub>1</sub>N<sub>1</sub>) type and B type viruses (Table 3).

TABLE 2. *Effect of immunization of mice with A/Osaka/9/70(H<sub>3</sub>N<sub>2</sub>) on the effect of challenge with virulent A/FM1/47 (H<sub>1</sub>N<sub>1</sub>)*

Group No.	Immunization with A/Osaka/9/70 (H <sub>3</sub> N <sub>2</sub> )	Challenge dose of A/FM1/47 (H <sub>1</sub> N <sub>1</sub> ) HAU/ml	death/total	Grade of consolidation of lungs				
				1	2	3	4	5
1	Yes	500	0/5	0	0	0	1	2
2	Yes	50	0/5	1	0	0	0	0
3	Yes	5	0/5	0	0	0	0	0
4	Yes			0	0	0	0	0
5	No	500	3/5	4	1			
6	No	50	0/5	1	2	1	1	1
7	No	5	1/5	0	3	1	1	
8	No			0	0	0	0	

Immunization and challenge were done by inhalation for 3 min as described elsewhere (Kurimura et al., 1971). The passage levels of the viruses used were:

$$\begin{aligned} &A/FM1/47 (H_1N_1) \quad (E_{14}M_{53}E_4M_9E_6) \\ &A/Osaka/9/70 (H_3N_2) \quad (E_8) \end{aligned}$$

where E and M mean passages in fertile eggs or in mice, respectively. The concentration of A/Osaka/9/70 (H<sub>3</sub>N<sub>2</sub>) used for immunization was 10<sup>4.5</sup> EID<sub>50</sub>/0.1ml. Mice in groups 4 and 8 were sacrificed on the day of challenge.

TABLE 3. *Effect of immunization of mice with B/Osaka/3/71 on the effect of challenge with virulent A/FM1/47 (H<sub>1</sub>N<sub>1</sub>)*

Group No.	Immunization with B/Osaka/3/71	Challenge dose of A/FM1/47(H <sub>1</sub> N <sub>1</sub> ) EID <sub>50</sub> /0.1ml	death/total	Grade of consolidation of lungs					
				1	2	3	4	5	6
1	Yes	1 × 10 <sup>5</sup>	5/6	3					
2	Yes	1 × 10 <sup>4</sup>	2/6	3	2	2	2	1	
3	Yes	1 × 10 <sup>3</sup>	1/6	1	1	0	1	0	
4	Yes			0	0	0	0	0	
5	No	1 × 10 <sup>5</sup>	5/6	1					
6	No	1 × 10 <sup>4</sup>	3/6	2	0	2			
7	No	1 × 10 <sup>3</sup>	1/6	1	1	2	1	1	
8	No			0	0	0	0	0	0

The passage levels of the viruses were B/Osaka/3/71 (E<sub>6</sub>) and A/FM1/47(H<sub>1</sub>N<sub>1</sub>) (E<sub>14</sub>M<sub>53</sub>E<sub>4</sub>M<sub>9</sub>E<sub>6</sub>) and the virus titer of B/Osaka/3/71 was 10<sup>6</sup> EID<sub>50</sub>/0.1 ml. Mice in groups 4 and 8 were sacrificed on the day of challenge. The antibody response to B/Osaka/3/71 was good judging from the HI antibody titer of the serum.

TABLE 4. *Effect of immunizations of mice with A/Okuda/57(H<sub>2</sub>N<sub>2</sub>) by intraperitoneal injection and inhalation on the effect of challenge with virulent A/FM1/47(H<sub>1</sub>N<sub>1</sub>)*

Group No.	Immunization	Challenge dose of A/FM1/47 (H <sub>1</sub> N <sub>1</sub> ) HAU/ml	deaths/total	Grade of consolidation of lungs							
				1	2	3	4	5	6	7	8
I	1	64	3/7	1	1	3	3				
	2	6.4	2/7	1	1	1	2	2			
	3	0.64	1/7	1	2	0	0	2	2		
	4			0	0	0	0	0	0	0	
II	1	64	2/8	1	1	1	2	0	2		
	2	6.4	0/8	0	1	1	0	2	0	4	1
	3	0.64	0/7	1	3	1	0	0	1	0	
	4			0	0	0	0	0	0	0	
III	1	64	8/8								
	2	6.4	4/8	2	4	3	2				
	3	0.64	2/7	1	1	1	1	3			
	4			0	0	0	0	0	0	0	

The amount of A/Okuda/57(H<sub>2</sub>N<sub>2</sub>) used for immunization by intraperitoneal injection was  $2 \times 10^6$  EID<sub>50</sub>/0.2 ml per mouse and that for inhalation for 3 min was  $10^6$  EID<sub>50</sub>/0.1 ml. The passage level of A/FM1/47(H<sub>1</sub>N<sub>1</sub>) was as for table 2 and that of A/Okuda/57(H<sub>2</sub>N<sub>2</sub>) was E<sub>285</sub>. Challenge was done by the inhalation method. Mice in groups I-4, II-4 and III-4 were sacrificed on the day of challenge to examine the extent of consolidation of their lungs and the antibody titers of their sera against A/Okuda/57(H<sub>2</sub>N<sub>2</sub>) and A/FM1/47(H<sub>1</sub>N<sub>1</sub>). Mice in these groups showed no lung consolidation and no HI antibody to A/FM1/47(H<sub>1</sub>N<sub>1</sub>).

TABLE 5. *Antibody response of mice after multiple exposures to live A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>)*

Group No.	No. of Exposures	Antibody Titer					
		A/Okuda/57 (H <sub>2</sub> N <sub>2</sub> )		A/FM1/47 (H <sub>1</sub> N <sub>1</sub> )		A/Osaka/9/70 (H <sub>3</sub> N <sub>2</sub> )	
		NT	HI	NT	HI	NT	HI
1	1	2 <sup>6.2c</sup> (5) <sup>d</sup>	2 <sup>8.0</sup> (4)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (4)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (4)
2	2	2 <sup>8.0</sup> (4)	2 <sup>8.2</sup> (6)		<2 <sup>3.0</sup> (6)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (6)
3	3	2 <sup>8.0</sup> (4)	2 <sup>7.7</sup> (6)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (6)	<2 <sup>3.0</sup> (4)	<2 <sup>3.0</sup> (6)
4	4	>2 <sup>8.0</sup> (5)	2 <sup>8.5</sup> (6)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (6)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (6)
5	5	>2 <sup>6.5</sup> (6)	2 <sup>9.5</sup> (2)	<2 <sup>3.0</sup> (6)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (2)
6 <sup>a</sup>	none	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (3)
7 <sup>b</sup>	none	<2 <sup>3.0</sup> (4)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (4)	<2 <sup>3.0</sup> (3)	<2 <sup>3.0</sup> (2)	<2 <sup>3.0</sup> (3)

NT and HI antibody titers in mouse sera were examined after single or multiple exposures to A2/Okuda/57 (H<sub>2</sub>N<sub>2</sub>) (E<sub>285</sub>,  $10^{6.5}$ – $10^{8.0}$  EID<sub>50</sub>/0.1 ml) by inhalation for 3 min. Mice were sacrificed by exsanguination 2 weeks after the last immunization. No mice showed any consolidation of the lung.

a Sacrificed at start of experiment

b Sacrificed at end of experiment

c Geometric mean titer

d Number of sera tested

This confirms previous results which excluded the possibility of interference as a cause of cross immunity.

Injection is a more convenient method of immunization than inhalation. Table 4 shows the result of immunization by the intraperitoneal route. Mice were preimmunized by intraperitoneal injection of live avirulent virus (A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>) and 14 days later, they were challenged with virulent A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) by the inhalation procedure. Intraperitoneal immunization also resulted in some protection against infection with a heterotypic virus via the respiratory tract. These results seem to agree with those of Waldman et al. (1968).

Henle and Lief (1963) reported that repeated infection of guinea pigs with one strain of influenza virus broadened their antibody spectra. We carried out a similar type of experiment to examine the nature of the pheno-

menon of broadened protection. Single or multiple exposures (at weekly intervals) of mice to live A/Okurda/57 (H<sub>2</sub>N<sub>2</sub>) by inhalation did not result in the appearance of HI antibody or NT antibody against A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) or A/Osaka/9/70 (H<sub>3</sub>N<sub>2</sub>) in the serum (Table 5). Similar results were obtained when mice were injected subcutaneously with doses of  $2 \times 10^{7.5}$  EID<sub>50</sub> per mouse, as shown in Table 5. We could not demonstrate the participation of serum neutralizing antibody in broadening the spectrum of protection by the above experiments, so we made the experiment shown in Table 6. However we could not detect any activity to neutralize A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) virus in sera or lung exudates of mice immunized two weeks previously with A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>), even when concentrated samples of sera or lung exudates were examined.

TABLE 6. *Neutralizing antibody in pooled and concentrated sera and lung exudates*

Group No.	No. of mice	Immunization with A/Okuda/57(H <sub>2</sub> N <sub>2</sub> )	Specimen	Times concentrated	NT titer of concentrated specimen	
					A/Okuda/57(H <sub>2</sub> N <sub>2</sub> )	A/FM1/47(H <sub>1</sub> N <sub>1</sub> )
1	23	Yes	serum	5.0	400	<2
			lung exudate	3.0	16	<2
2	23	No	serum	3.3	<8	<2
			lung exudate	4.0	<8	<2

*10<sup>8.5</sup> EID<sub>50</sub>/0.1 ml of A/Okuda/57(H<sub>2</sub>N<sub>2</sub>) (E<sub>288</sub>) was used for immunization by inhalation. Two weeks after immunization the sera and lung washings of mice in each group were pooled and concentrated as described in "Materials and Methods". Four or five fertile eggs were used to test for NT antibody at each dilution of concentrated sera or concentrated lung exudates.*

## DISCUSSION

Cross protection or broadening of the spectrum of immunity to type A influenza viruses is a very important phenomenon for evaluation of the value of live influenza vaccine. The present experiments indicate that immunization of mice with avirulent A (H<sub>2</sub>N<sub>2</sub>) or A (H<sub>3</sub>N<sub>2</sub>) influenza viruses results in protection against the virulent strain A/FM1/47 (H<sub>1</sub>N<sub>1</sub>). More-

over immunization with A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>) results in protection against virulent A/Osaka/5/70 (H<sub>3</sub>N<sub>2</sub>) (Maeda, unpublished data). Cross protection is only observed within the type A group and does not occur between type A and type B viruses. Thus, cross immunity is possibly caused by humoral antibody. However, so far it has not been possible to demonstrate neutralizing antibody in lung secretions or sera

and even after multiple immunization of mice with live A/Okuda/57(H<sub>2</sub>N<sub>2</sub>), no neutralizing antibodies against A/FM1/47(H<sub>1</sub>N<sub>1</sub>) or A/Osaka/9/70 (H<sub>3</sub>N<sub>2</sub>) could be found in the serum or lung exudate. Moreover, pooled and concentrated sera and washing fluid from the lungs of mice immunized with A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>), showed no neutralizing activity against A/FM1/47 (H<sub>1</sub>N<sub>1</sub>). The possible presence of antibody should be examined using a more sensitive method, e.g. the plaque reduction method, and neutralizing activity should be tested using more concentrated washing fluid from the lungs.

Secretory IgA is the most important factor in protection against viral respiratory diseases (Mann et al., 1968; Rossen et al., 1971). We could not demonstrate neutralizing antibody against A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) in lung washings, but there are two reports which support the possibility that IgA is the cause of cross protection. Waldman et al. (1970) suggested that IgA is less specific than IgG in lung secretions and this could be so in our mice. In their experiments, the post-immunization NT antibody titers to homotypic virus A/Taiwan/64 (H<sub>2</sub>N<sub>2</sub>) of the sputum were between 1:2 and 1:32, while those to heterotypic A/Hong Kong/68 (H<sub>3</sub>N<sub>2</sub>) or A/PR/8(H<sub>0</sub>N<sub>1</sub>) virus were between 1:1 and 1:4. Three of 17 cases did not show NT antibody against heterotypic type A viruses, but it is conceivable that, if the

antibody level in the sputum against homotypic virus were about 1:2 to 1:32 (which correspond to values of 4 to 64 in this paper), it would probably be possible to detect neutralizing activity against heterotypic viruses. As shown in Table 6, NT antibody to heterotypic virus could not be demonstrated in pooled and concentrated sera though there was a fairly high level of neutralizing activity of homotypic virus. Intraperitoneal injection of live A/Okuda/57 (H<sub>2</sub>N<sub>2</sub>) virus also induced cross protection against A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) in mice but the extent of protection was somewhat less than when the inhalation procedure was used. This also seems to agree with the results of Waldman et al. (1968). Another way to evaluate the role of secretory IgA in cross protection would be to employ a different route of challenge, but, as already reported (Hoyle, 1968; Schmidt-Ruppin, 1968), challenge of mice with A/FM1/47 (H<sub>1</sub>N<sub>1</sub>) by the intraperitoneal route was unsuccessful.

This phenomenon of cross protection among type A influenza viruses shows some degree of specificity, so its cause may differ from that of the phenomenon of non-specific protection between rhinovirus and Cocksackie virus (Cate et al., 1969).

Studies on the possibility that some cell associated factor is involved in cross protection among type A influenza viruses are in progress.

## REFERENCES

- Cate, T. R., R. G. Douglas, Jr., and R. B. Couch. 1969. Interferon and resistance to upper respiratory virus illness. *Proc. Soc. Exp. Biol. Med.* 131: 631-636.
- Henle, W., and F. S. Lief. 1963. The broadening of antibody spectra following multiple exposures to influenza viruses. *Amer. Rev. Resp. Dis.* 88: 379-386.
- Hoyle, L. 1968. *Virology Monographs*. "The Influenza Viruses" Springer-Verlag, New York Inc.
- Kurimura, T., A. Hirano, N. Suzuki, and Y. Okuno. 1971. Influenza virus infection in mice: the effect of avirulent virus infection on the pathogenicity of highly virulent virus. *Biken J.* 14: 65-73.
- Mann, J. J., R. H. Waldman, Y. Togo, G. G. Heiner, A. T. Dawkins, and J. A. Kasel. 1968. Antibody response in respiratory secretions of volunteers given live and dead influenza virus. *J. Immunol.* 100: 726-735.
- Nakamura, K. 1965. Pathogenicity and immunogenicity of various strains of influenza virus for mice. *Biken J.* 8: 155-165.

- Okuno, Y., and K. Nakamura. 1966. Prophylactic effectiveness of live influenza vaccine in 1965. *Biken J.* 9: 89-95.
- Rossen, R. D., J. A. Kasel, and R. B. Couch. 1971. The secretory immune system: Its relation to respiratory viral infection. *Prog. Med. Virol.* 13: 194-238.
- Schmidt-Ruppin, K. H. 1968. Intraperitoneal infection of Ehrlich ascites tumor mice with viruses of low virulence in vivo, a new chemotherapeutic test system. *Arch. Ges. Virusforsch.* 25: 126-128.
- Stuart-Harris, C. H. 1970. Control of influenza. *Arch. Environ. Health.* 21: 276-285.
- Waldman, R. H., J. A. Kasel, R. V. Fulk, Y. Togo, R. B. Hornick, G. G. Heiner, A. T. Dawkins, Jr., and J. J. Mann. 1968. Influenza antibody in human respiratory secretions after subcutaneous of respiratory immunization with inactivated virus. *Nature* 218: 594-595.
- Waldmann, R. H., F. M. Wigley, and P. M. Small, Jr. 1970. Specificity of respiratory secretion antibody against influenza virus. *J. Immunol.* 105: 1477-1483.
- Willers, H., and A. S. Beare. 1970. Laboratory characteristics of influenza A viruses attenuated for man. *Arch. Ges. Virusforsch.* 30: 187-194.