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INFLUENZA VIRUS INFECTION IN MICE: THE EFFECT OF AVIRULENT VIRUS INFECTION ON THE PATHOGENICITY OF HIGHLY VIRULENT VIRUS

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SUMMARY Coinfection of mice with an avirulent (A2/Okuda/57) and a highly virulent (A1/FM1/47) influenza virus enhanced the virulence of the latter. This enhancement was observerd even when UV-irradiated A2/Okuda/57 was used for coinfection.

On the contrary, when mice were preimmunized with live A2/Okuda/57, they showed resistance to challenge with virulent A1/FM1/47.

Possible explanations of these phenomena are discussed.

INTRODUCTION

Several factors protect animals against infection. These are specific antibody, interferon and unknown factors (Cate et al., 1969). When animals are simultaneously or successively infected with two strains of virus, interference, complementation and other phenomena might be observed.

The effect of live influenza vaccine has already been reported by several authors (Beare et al., 1968; Okuno, 1963; Okuno and Nakamura, 1966). It was found that, live influenza vaccine is more effective than killed vaccine. Antigenic variation of influenza virus is the most troublesome problem in development of influenza vaccine, and reduction of protection with continued antigenic drift has also been shown with a mouse-influenza system (Kaye et al., 1969). The effectiveness of immunization of experimental animals with live influenza virus in preventing infection with different types of influenza virus has been reported (Francis and Magill, 1938; Henle and Lief, 1963). Accordingly we chose the mouse-influenza virus system to investigate various fundamental problems about live influenza vaccine. Natural influenza virus infection starts in the epithelium of the respiratory tract, so inhalation was used as the inoculation method. Different results were obtained on varying the times of inoculation of a virulent and an avirulent virus strains.

The viruses used in this work were A2/Okuda/57 and A1/FM1/47. The virulence of A2/Okuda/57 and A1/FM1/47 in mice has

been discussed thoroughly by Nakamura (1965). A2/Okuda/57 induces high titers of HI and neutralizing antibody in mice without killing the animals or consolidating their lungs. On the other hand, A1/FM1/47 is highly virulent in mice inducing consolidation of the lungs and when a high titer of virus is used for inhalation, it is lethal.

MATERIALS AND METHODS

1. *Mice*

Four to 5 week old inbred female mice of the ddO strain were used. Experimental groups were separated from each other in ventilated metal cabinet compartments.

2. *Influenza virus*

A2/Okuda/57 and A1/FMI/47 (Nakamura, 1965) were used. Ten day old fertile eggs were infected with the viruses and two days later the allantoic fluid was harvested and centrifuged. The supernatant was used for infection of mice. For dilution, LE medium (Earle's solution containing lactalbumin hydrolysate) was used as diluent. The virus titer was determined by hemagglutination (HA) of chick red blood cells or by the EID_{50}.

3. *Infection or immunization of mice*

For infection or immunization of mice, the inhalation method was adopted (Nakamura, 1965). A nebulizer with an air compressor (6 liters per min) was used. A group of mice (4 to 6 mice) were put in a glass vessel with a narrow outlet and virus was blown into the vessel for 3 min. Mice were observed for two weeks after infection. Then, surviving mice were sacrificed by exsanguination by cardiac puncture and consolidation of the lungs was carefully checked. Sera of surviving mice were used for the hemagglutination inhibition (HI) test and neutralization (NT) test.

4. *Hemagglutination inhibition (HI) test*

Mouse sera were treated with an equal volume of RDE (Takeda Pharmaceutical Co.) overnight and then heated at 56 C for 30 min. For assay, the microtiter method was used. Serial two fold dilutions of sera were mixed with an equal volume of virus (equivalent to 4 HA units) and the mixture was incubated at 37 C for 30 min. Then, two volumes of 5% chick red blood cells were added. The antibody titer is expressed as the power of 2 of the reciprocal of the highest dilution of serum causing detectable inhibition of hemagglutination.

5. *Neutralization test*

The neutralizing antibody titer against A1/FMI/47 in mouse sera or in lung exudates was determined as described elsewhere (Nakamura, 1965). Aliquots of 0.4 ml of serially diluted sera were mixed with 0.4 ml of A1/FMI/47 virus (100 EID_{50}/0.1 ml) and incubated at 37 C for 1 hr. After incubation, 0.2 ml aliquots of each mixture were inoculated into 3 eggs. After 2 days, agglutination of chick red blood cells by the allantoic fluid was examined. The antibody titer is expressed as the reciprocal of the highest serum dilution showing neutralization of the virus in more than 50% of the eggs.

6. *Recovery of virus from infected lung*

Growth of A2/Okuda/57 in mouse lung was followed after infection of mice by the inhalation method. Mice were sacrificed by bleeding and the lungs were taken out together with the trachea. Then each lung was washed with 0.5 ml of sterile phosphate buffered saline and the washing fluid was collected in a sterile tube. After one cycle of freezing and thawing, the lung was homogenized in a mortar, and 5 ml of Hanks' BSS containig 0.025% BSA, kanamycin (50 μg/ml) and amphotericin B (2 μg/ml) were added to the homogenate. Homogenized samples were centrifuged at 10,000 rev/min for 30 min, and the supernatant was used for titration of virus. All the reisolated virus was identified as A2/Okuda/57 using homotypic and heterotypic anti-sera. The lung exudate was assayed for interferon activity and neutralizing antibody.

7. *Interferon assay*

For interferon assay, sera or lung exudates were treated as described by Mirchamsy and Rapp (1969). Mouse L cell monolayers (60 mm petri dish) were treated with 0.5 ml of serially diluted specimens for six hours at 37 C in a CO_2-incubator. After sucking off the liquid, 0.1 ml of Mengo virus containing 50–100 PFU was added and incubation was continued at 37 C for 1 hr. Then the petri dishes were overlaid by 199 medium containing 5% calf serum and 1% Bacto-agar, and incubated at 37 C for 2 or 3 days before counting the plaques. The interferon titer is expressed as the reciprocal

of the highest dilution of the sample which caused more than 50% plaque reduction.

8. *UV-irradiation of virus*

Infected allantoic fluid was diluted 1 : 5 with phosphate-buffered saline and 3 ml aliquots of the diluted material were put in 60 mm plastic petri dishes. These petri dishes were placed under a germicidal lamp (Toshiba germicidal lamp GL-15) at a distance of 30 cm. During irradiation, petri dishes were shaken by hand.

RESULTS

1. *Coinfection with two virus strains*

The virulent strain A1/FM1/47 and avirulent strain A2/Okuda/57 were used throughout for coinfection. Table 1 shows the result of coinfection of mice with A2/Okuda/57 and A1/FM1/47. The A2/Okuda/57 strain caused no death or consolidation of the lungs (Gr. 1), while A1/FM1/47 caused death in one of five animals (Gr. 4). Coinfection of mice with A1/FM1/47 and A2/Okuda/57 resulted in a higher incidence of death and consolidation of the lungs (Gr. 2 and 3). There was not much difference between the HI antibody (anti-A1/FM1/47) titers of sera in the group infected with A1/FM1/47 (Gr. 4) alone and in those coinfected with both A1/FM1/47 and A2/Okuda/57 (Gr. 2 and 3).

To confirm the results in Table 1, various dilutions of the FM1 strain were used for inhalation with or without a constant amount of the A2/Okuda/57 strain. The results in Table 2 also indicate that coinfection with the A2/Okuda/57 strain enhances the virulence of the FM1 strain judging from the incidence of death and consolidation of the lungs. This suggests that, the A2/Okuda/57 strain supports or complements growth of the A1/FM1/47 strain in mice to some extent. To investigate this, the A2/Okuda/57 strain was subjected to UV-irradiation and then used for coinfection (Table 3). UV-irradiation should minimize the possiblity of complementation. Even after UV-irradiation for 5, 10 or 15 min, the A2/Okuda/57 strain still enhanced the virulence of the A1/FM1/47 strain on coinfection.

TABLE 1. *Coinfection of mice with A1/FM1/47 and A2/Okuda/57 (1)*

Group No.	Virus	Virus concentration (HAU/ml)	Death/ total	Consolidation/ survived	HI titer											
					A1/FM1/47						A2/Okuda/57					
					1	2	3	4	5	6	1	2	3	4	5	6
1	A2/Okuda/57	50	0/6	0/6	<3	<3	<3	<3	<3	<3	6	<3	4	5	5	4
2	A2/Okuda/57	50	5/6	1/1	9						3					
	A1/FM1/47	10														
3	A2/Okuda/57	5	3/6	3/3	9	10	—				<3	<3	—			
	A1/FM1/47	10														
4	A1/FM1/47	10	1/5	3/4	8	9	9	7			<3	<3	<3	<3		
5	LE medium	0	0/5	0/5	<3	<3	<3	<3	<3		<3	<3	<3	<3	<3	

Five week old female ddO mice were used. After inhalation of virus, mice were observed for 14 days, and survived mice were bled and checked for consolidation of the lung and HI titer of the sera. Concentration of the virus is expressed in HA units per ml. The passage history of viruses are shown below.

A1/FM1/47 (E_{14} M_{53} E_4 M_9 E_2)

A2/Okuda/57 (E_{281})

where E or M mean passages in egg or in mouse.

HI titer of serum is expressed as $\log_2$ *of the reciprocal of the highest dilution of serum which inhibits completely the hemagglutination by the virus equivalent to 4 HAU.*

" — " *means "not tested" because of failure of bleeding.*

TABLE 2. *Coinfection of mice with A1/FM1/47 and A2/Okuda/57 (2)*

Group No.	Virus	Virus concentration		Death/total	Consolidation/ survived
		HAU/ml	EID_{50}/0.1 ml		
1	A2/Okuda/57	25	5×10^6	0/4	0/4
2	A2/Okuda/57	25	5×10^6	1/5	4/4
	A1/FM1/47	2	1.5×10^5		
3	A2/Okuda/57	25	5×10^6	1/5	0/4
	A1/FM1/47	0.2	1.5×10^4		
4	A2/Okuda/57	25	5×10^6	0/5	0/5
	A1/FM1/47	0.02	1.5×10^3		
5	A1/FM1/47	20	1.5×10^6	4/4	
6	A1/FM1/47	2	1.5×10^5	1/5	1/4
7	A1/FM1/47	0.2	1.5×10^4	0/5	0/5
8	A1/FM1/47	0.02	1.5×10^3	0/5	0/5

All the numbers are expressed as indicated in the legend of Table 1. The passage history of the viruses are A1/FM1/47 (E_{14} M_{53} E_4 M_9 E_3) and A2/Okuda/57 (E_{282}).

TABLE 3. *Coinfection of mice with A1/FM1/47 and UV-irradiated A2/Okuda/57*

Group No.	Virus	Virus concentration EID_{50}/0.1 ml	Death/total	Consolidation/ survived
1	A1/FM1/47	5×10^4	0/4	2/4
2	A1/FM1/47	5×10^4	2/4	2/2
	A2/Okuda/57	2.5×10^7		
3	A1/FM1/47	5×10^4	1/4	3/3
	A2/Okuda/57 (UV 5 min)	$< 2.5 \times 10^2$		
4	A1/FM1/47	5×10^4	3/4	1/1
	A2/Okuda/57 (UV 10 min)	$< 2.5 \times 10$		
5	A1/FM1/47	5×10^4	1/4	3/3
	A2/Okuda/57 (UV 15 min)	< 2.5		
6	A2/Okuda/57	2.5×10^7	0/4	0/4
7	A2/Okuda/57 (UV 10 min)	$< 2.5 \times 10$	0/4	0/4

Passage history of the viruses used are A2/Okuda/57 (E_{282}) and A1/FM1/47 (E_{14} M_{53} E_4 M_9 E_4). The condition of UV-irradiation is shown in Materials and Methods. The absolute amount of A2/Okuda/57 is constant irrespective of UV-irradiation.

2. *Preimmunization of mice*

The above results show that coinfection of mice with an avirulent and a virulent strain resulted in a higher mortality and higher incidence of consolidation of the lungs. Therefore, the effect of preimmunization of mice with A2/Okuda/57 on challenge with A1/FM1/47 was examined. Table 4 shows the results. Mice in groups 1, 2, 3 and 4 were preimmunized with A2/Okuda/57 by the inhalation method. Groups 5 to 8 were controls which were not immunized. Nine days after preimmunization, groups 1, 2, 3, 5, 6 and 7 were challenged with A1/FM1/47 by inhalation and then all groups were observed for 14 days. Mice in groups 4 and 8 were sacrificed by cardiac puncture on the day of challenge and the antibody titer of their sera was measured. All the preimmunized groups showed enhanced resistance to challenge with A1/FM1/47, while in the control groups death occurred at all three challenge levels. The protective effect of immunization against challenge virus is evident even though the antigenic types (A1 and A2) were different. This can also be concluded from the incidence of consolidation of the lungs. A similar type of experiment was performed to confirm these results. The interval between preimmunization and challenge was lengthened to 22 days and a higher challenge dose of A1/FM1/47 was used, because at the time of challenge mice were 8 weeks old and somewhat resistant to challenge virus. The results in Table 5 again show that preimmunization with A2/Okuda/57 resulted in a remarkable protective effect against challenge virus. On the day of challenge, no neutralizing antibody to A1/FM1/47 was found in the sera of mice which had been preimmunized with A2/Okuda/57 (Table 6).

TABLE 4. *The effect of preimmunization of mice with A2/Okuda/57 on the challenge with virulent A1/FM1/47 (1)*

Group No.	Immunization with A2/Okuda/57	Challenge dose of A1/FM1/47 EID_{50}/0.1 ml	Death/ total	Consolidation/ survived
1	Yes	6×10^4	0/5	2/5
2	Yes	1.5×10^4	0/6	1/6
3	Yes	3×10^3	0/5	0/5
4	Yes			
5	No	6×10^4	1/5	4/4
6	No	1.5×10^4	2/5	1/3
7	No	3×10^3	1/5	1/4
8	No			

Both preimmunization and challenge were done by inhalation for 3 min as described in Materials and Methods. Cahllenge was done 9 days after preimmunization of mice. The titer of A2/Okuda/57 used for preimmunization was 6×10^7 EID_{50}/ml. 6×10^4 EID_{50} of A1/FM1/47 corresponds to 5×10^3 PFU assayed on one day old monolayer of chick embryo cells. Passage history of the viruses is the same as in Table 3.

A similar experiment was done using live or UV-killed A2/Okuda/57 virus or formalin-killed A1/FM1/47 virus for immunization. Immunization was done by inhalation. Twelve days after immunization these mice were challenged with A1/FM1/47. Only live A2/Okuda/57 virus induced a protective effect against A1/FM1/47 (Table 7).

The mechanism of protection against heterotypic virulent virus can be explained in several ways. The first possiblity is interference between preexisting A2/Okuda/57 virus and challenging A1/FM1/47 virus. Fig. 1 shows the growth of the A2/Okuda/57 strain in mouse lung. After infection of mice by inhalation, the A2/Okuda/57 virus replicates for a few days and then the virus titer declines gradually. Eleven days after infection, there was no detectable virus in the lungs. Since we challenged on the 9th, 12th or 22nd day after immunization, the possibility of interference between the two virus strains seems rather improbable. The second possibility is that interferon is the cause of resistance. In these experiments, we could not detect interferon activity in mouse sera. We detected interferon

TABLE 5. *The effect of preimmunization of mice with A2/Okuda/57 on the challenge with virulent A1/FM1/47 (2)*

Group No.	Immunization with A2/Okuda/57	Challenge dose of A1/FM1/47 EID_{50}/0.1 ml	Death/ total	Consolidation/ survived	HI titer									
					A1/FM1/47					A2/Okuda/57				
					1	2	3	4	5	1	2	3	4	5
1	Yes	4.7×10^6	3/5	2/2	8	9				6	6			
2	Yes	4.7×10^5	0/5	2/5	8	8	7	7	8	6	6	7	6	7
3	Yes	4.7×10^4	0/5	1/5	7	9	7	7	8	5	7	8	6	6
4	Yes				<3	<3	<3	<3	<3	8	4	6	6	6
5	No	4.7×10^6	5/5											
6	No	4.7×10^5	1/4	3/3	9	8	8			<3	<3	<3		
7	No	4.7×10^4	3/5	2/2	9	8				<3	<3			
8	No				<3	<3	<3	<3	<3	<3	<3	<3	<3	<3

Both preimmunization and challenge were done by inhalation for 3 min as described in Materials and Methods. Challenge was done 22 days after preimmunization of mice. The titer of A2/Okuda/57 used for preimmunization was 10^9 EID_{50}/ml. Passage history of the viruses is the same as in Table 3.

TABLE 6. *Neutralizing antibody against A1/FM1/47 in mouse sera*

Group No.	Mouse No.	Immunization with A2/Okuda/57	Challenge dose of A1/FM1/47 EID_{50}/0.1 ml	NT antibody titer
2	2-2	Yes	4.7×10^5	>64
3	3-1	Yes	4.7×10^4	>64
	3-2			>64
4	4-3	Yes	—	< 8
	4-4			< 8
6	6-1	No	4.7×10^5	>64
7	7-1	No	4.7×10^4	>64
	7-2			>64
8	8-2	No	—	<16
	8-5			<16

Experimental conditions are shown in the legend to Table 5.

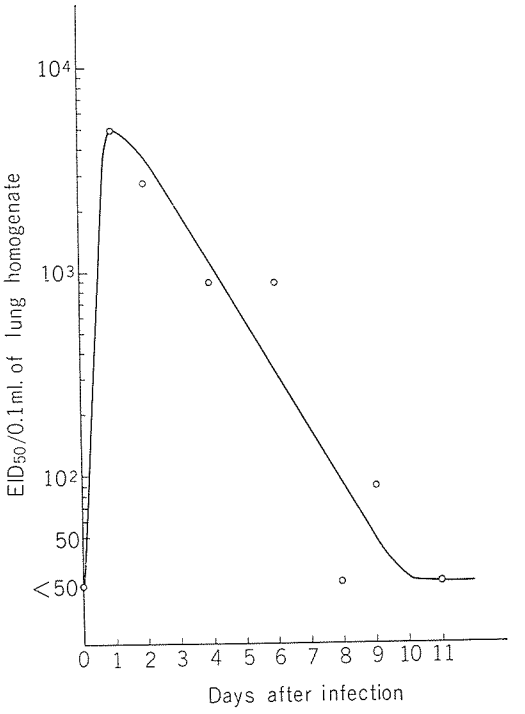

TABLE 7. *The effect of preimmunization of mice with live A2/Okuda/57, UV-irradiated A2/Okuda/57 and formalin-killed A1/FM1/47 on the challenge with virulent A1/FM1/47*

Group No.	immunization	Challenge dose of A1/FM1/47 EID_{50}/0.1 ml	Death/ total	Consolidation/ survived	Extent of consolidation[a] 1	2	3	4
1	A2/Okuda/57	1×10^6	0/4	2/4	−	−	+	+
2	A2/Okuda/57	1×10^5	0/4	1/4	−	−	−	+
3	A2/Okuda/57	1×10^4	0/4	2/4	−	+	+	−
4	A2/Okuda/57 (UV 5 min)	1×10^6	4/4					
5	A2/Okuda/57 (UV 5 min)	1×10^5	3/4	1/1	+			
6	A2/Okuda/57 (UV 5 min)	1×10^4	1/4	2/3	−	++	+	
7	No	1×10^6	1/4	3/3	+	++	+	
8	No	1×10^5	1/4	3/3	+	++	+	
9	No	1×10^4	0/4	3/4	−	++	++	++
10	A1/FM1/47 (Formalin-killed)	1×10^6	4/4					
11	A1/FM1/47 (Formalin-killed)	1×10^5	0/4	4/4	+	++	++	++
12	A1/FM1/47 (Formalin-killed)	1×10^4	0/4	3/4	+	−	++	+

a −: without consolidation.
+: slight.
++: moderate to severe.

Both preimmunization and challenge were done by inhalation for 3 min as described in Materials and Methods. Challenge was done 9 (Gr. 10, 11, 12) or 12 (Gr. 1 to 6) days after preimmunization of mice. The titer of A2/Okuda/57 used for preimmunization was 6.4×10^6 EID_{50}/ml. Passage history of the viruses used are A2/Okuda/57 (E_{284}) and A1/FM1/47 (E_{14} M_{53} E_4 M_9 E_4). The condition of UV-irradiation is shown in Materials and Methods. The procedure to prepare formalin-killed vaccine was as follows. One hundred and eighty ml of infected chorioallantoic fluid was centrifuged (Spinco L, 30,000 rev/min for 3 hours) and the pellet was resuspended with 10 ml of PBS⁻. To the virus suspension, 0.02 ml of formalin was added. The concentration of formalin-killed virus was 1024 HAU/ml.

◀

FIGURE 1. *Virus growth in mouse lung after inhalation of A2/Okuda/57.*

Mice were infected with freshly prepared A2/Okuda/57 (E_{285}, 256 HAU/ml) by inhalation for 3 min. Virus titer is expressed as the geometric mean of two mice.

activity in lung exudates on the first or second day after immunization but not after the sixth day (Fig. 2). This excludes the possiblity that interferon is involved in the protection. Moreover, we could not detect neutralizing antibody to A1/FM1/47 in lung secretions after immunization of mice with A2/Okuda/57.

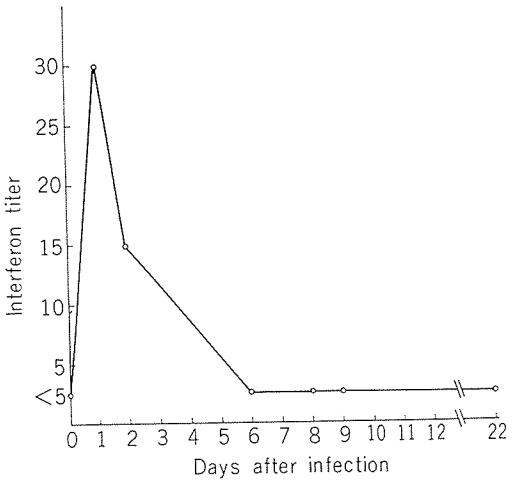

FIGURE 2. *Interferon activity in mouse lung secretion after infection of A2/Okuda/57 by inhalation.*
This experiment was done using the same mice which were used for viral growth shown in Fig. 1.

DISCUSSION

The problems of control of viral infections in man have been thoroughly reviewed (Hillman, 1969). The present work was on some fundamental problems related to live influenza vaccine.

The enhancement of virulence observed on coinfection with a virulent and an avirulent influenza virus can be explained in several ways. These are: (a) complementation, (b) inhibition of interferon synthesis as the result of inhibition of host cell protein synthesis (Gandhi and Burke, 1970) by infection of A2/Okuda/57, (c) supply of some viral activity such as sialidase activity from the avirulent strain and (d) an unknown factor(s). The first possibility, which postulates that the viral genome of A2/Okuda/57 will provide some function for A1/FM1/47, seems improbable, since, even after 15 min UV-irradiation the former strain enhanced the virulence of the latter strain. However it is possible that A2/Okuda/57 became more virulent on acquisition of some function from A1/FM1/47 by complementation. Judging from the HI titer of the sera, the viral replication of A2/Okuda/57 strain was not enhanced, so this possiblity seems rather unlikely. Ten or 15 min irradiation with ultraviolet light should reduce the ability of virus to induce interferon. However, mortality and the incidence of lung consolidation were similar in the groups coinfected with UV-irradiated A2/Okuda/57 virus and live A2/Okuda/57 virus. Neyertheless explanation (b) is still possible. Explanation (c) seems possible, because proteins are much more resistant than ribonucleic acid to UV-irradiation. However it is uncertain whether the amount of A2/Okuda/57 used for inhalation was sufficient to provide sialidase activity for A1/FM1/47 at the time of infection of host cells in vivo.

The protective effect of preimmunization with a heterotypic virus can be explained in several ways. Antibodies with a broad spectrum have been shown to be evoked by infection with live influenza virus in guinea pigs (Henle and Lief, 1963) and mice (Francis and Magill, 1938). Thus the data in Tables 4, 5 and 7 showing that immunization with a different type of virus protects mice against a virulent strain may be because of antibody (IgA or IgG) in secretions from the respiratory tract or in the serum. A second explanation is that interferon may cause resistance (Gandhi and Burke, 1970). This possibility seems unlikely, because 9 or 22 days after infection of mice with influenza virus, there is probably little interferon activity in the sera or respiratory secretions (Cate et al., 1969). Moreover we found no interferon activity in secretions of the lungs or in the serum at the time of challenge. A third explanation is that factors other than interferon cause interference. To investigate this possibility, the growth of the A2/Okuda/57 strain in mouse lung was investigated. The viral growth curve showed that there was little, if any, A2/Okuda/57 virus in mouse lung at the time of challenge with A1/FM1/47. A fourth possibility is the presence of some unknown factor. Cate et al. (1969) reported the presence of this type of factor(s) in cases of rhinovirus

and coxsackie virus infection. In any case, the results shown in Tables 4, 5 and 7 are encouraging for development of live influenza vaccine which can overcome the variation in antigenicity of influenza virus. The fact that inactivated influenza vaccine (A2/Japan/305/57) was not so effective against the 1967 A2 strain (Kaye et al., 1969), also, suggests the importance of live influenza vaccine.

Studies are required on whether these phenomena are observed with all strains of influenza virus.

The problems discussed above are now under investigation.

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