BIKEN JOURNAL Vol. 13, 277-287, 1970

NEUTRALIZATION ANTIBODY RESPONSE TO A2 HONG KONG INFLUENZA VACCINE AND ITS DURATION IN SCHOOL AGE CHILDREN

HIROSHI HOSAI, SUGURU OMURA, AKIKO MAEDA and NOBUHARU KUNITA

Virus Laboratory, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-ku, Osaka

(Received May 30, 1970)

SUMMARY Bivalent inactivated vaccine (A2 Hong Kong and B type influenza vaccine) was administered to about 540 school children in an elementary school in Osaka City from October to November, 1968. Side reactions were checked after vaccination and the antibody responses to A2 Hong Kong and B viruses were measured by the neutralization test.

Six to 9 per cent of the vaccinees showed slight systemic reactions on the day of vaccination. A local reddening reaction was observed in a few percent of the children but this decreased the next day.

Neutralization antibody titers were generally 2 to 3-fold higher than hemagglutination inhibiting antibody titers. Against A2 Hong Kong virus, 74% and 84% of the children exhibited 4-fold or greater rises in the neutralization titer and hemagglutination inhibition titer, respectively. The rise in the antibody level of the vaccinees was considerably less than in a group of naturally infected children.

However, the degrees of decrease in the antibody titers of the vaccinees and infected children one year late, were similar in neutralization and hemagglutination inhibition; decreasing to 1/2 to 1/3 of the initial titers.

INTRODUCTION

The incidence of influenza is highest in children of school age. Thus children are important in dissemination of the virus and in initiation of epidemics. So it is important to vaccinate school children to prevent them from transmitting the virus to other social groups in epidemics. Accordingly, since 1963, it has become the policy of the Government to carry out mass administration of inactivated influenza vaccine yearly in Japan to all children of school age usually from the end of October to mid-November.

An influenza epidemic was expected in the winter of 1968 to 1969 in Japan as a major antigenic shift had been found in influenza viruses isolated in preceding years. The authors

carried out mass administration of the A2 Hong Kong influenza vaccine from the end of October to the beginning of November at an elementary school in Osaka City as a part of the vaccination programme, before the outbreak of the epidemics and studied the responses of children to the new type (A2 Hong Kong) vaccine.

The side reactions due to the vaccine were checked for the first three days after vaccination. The antibody levels in the vaccinated children were examined one month and one year after vaccination and compared with the corresponding titers in a group of children who contracted influenza.

MATERIALS AND METHODS

1. *Vaccine*

The bivalent vaccine supplied from Osaka Research Foundation for Microbial Diseases was composed of 200 CCA/ml of A2/Aichi/2/68 (Hong Kong type), 50 CCA/ml of B/Tokyo/7/66 and 50 CCA/ml of B/Tokyo/1/67 influenza viruses.

2. *Vaccine administration*

About 540 school children in an elementary school in Osaka City, of six to twelve years old, were given two subcutaneous injections of 0.3 ml of the vaccine, on October 21 and on November 6, 1968.

3. *Blood specimens from the vaccinees*

Blood specimens were taken twice, just before the first vaccination and four weeks after the second vaccination. After one year, on December 15, 1969, a further 20 blood specimens were obtained from the vaccinees.

The antibody titers of 191 paired sera and 20 triple-paired sera were tested for neutralization and hemagglutination inhibition antibodies. The titer against A2 type virus and the antibody response to B type influenza virus were both examined by the hemagglutination inhibition test.

4. *Blood specimens from a group of patients*

During the epidemics of Hong Kong influenza, from the end of November 1968 to the beginning of March 1969, about 70 blood specimens were obtained from patients (school children). Specimens were obtained twice, once in the acute and once in the convalescent period. The convalescent sera were usually taken two weeks after the onset of the disease. Twelve blood specimens were also obtained from these patients on December 14 or 15, 1969, one year after the onset of the disease. The antibody responses in these paired sera and triple-paired sera were compared with those in sera from the vaccinees.

5. *Neutralization (NT) test*

1) *Antigen*

One of the Hong Kong influenza virus strains, A2/Osaka/111/69, isolated during the epidemics using a secondary culture of monkey kidney (MK) cells, was used as the antigen. The virus had been passaged five times in MK cell cultures and stored at −70 C before use. The infectivity titers of the harvested cells were $10^{4.0}$ to $10^{4.75}$ $TCID_{50}$/0.1 ml in MK cells and $10^{4.5}$ to $10^{5.0}$ EID_{50}/0.1 ml in the chorioallantoic cavity of embryonated eggs.

2) *Conditions for the NT test*

Preliminary experiments were made on the optimal antigen doses and incubation time for the test. First, various amounts of A2/Osaka/111/69 virus were mixed with a constant amount of hyper-immune rabbit serum.

As shown in Table 1, when the virus dose was reduced to about one tenth, the NT titer increased 2 to 4 fold with virus doses of 100 to 1000 $TCID_{50}$/0.1 ml.

Next, a mixture of the hyper-immune serum and virus (100 $TCID_{50}$/0.1 ml) was kept at room temperature for various periods. It was found that for complete development of neutralization, a period of

TABLE 1. *Relation of viral dose to NT antibody titer*

Virus dose ($TCID_{50}$/0.1 ml)	NT titer
$10^{4.0}$	40
$10^{3.0}$	640
$5\times10^{2.0}$	1280
$10^{2.0}$	2560

Serial 2-fold dilutions of serum were tested at various virus dose. The neutralization was done at room temperature for one hour.

TABLE 2. *Relation of incubation time to NT antibody titer in the neutraliztion test*

Incubation time (Minutes)	NT titer
10	160
20	320
30	320
40	640
60	640
90	640
120	640
180	640

The neutralization reaction was done at room temperature. The serum employed was hyper-immune anti-A2/Osaka/52/69; A2 Hong Kong influenza virus. The virus dose was 100 $TCID_{50}$/0.1 ml.

more than 20 minutes was necessary, as shown in Table 2.

Based on these results, an incubation time of 60 minutes and a virus dose of 100 $TCID_{50}$/0.1 ml were used throughout for the NT test.

3) *Procedure*

All sera were heated to 56 C for 30 minutes before the tests. The inactivated sera were diluted serially two-fold with Hanks' BSS. Virus was added to each dilution and the mixtures were kept at room temperature for 60 minutes. Then the medium of MK cell cultures (15×95 mm tube) was removed, and 0.2 ml of the preincubated mixture was inoculated onto each culture. The cell cultures were kept at room temperature for one hour to allow virus adsorption and then 1.0 ml of medium 199 was added. Inoculated cultures were incubated at 37 C for 7 days until a cytopathogenic effect appeared in control tubes. NT antibody titers are expressed as the reciprocals of the maximum dilution of serum causing complete inhibition of the cytopathic effect.

6. *Hemagglutination inhibition (HI) test*

Three volumes of a solution of receptor destroying enzyme were added to one volume of serum to eliminate non-specific inhibitors. The mixture was incubated at 37 C overnight, and then heated at 56 C for 30 minutes to inactivate the enzyme.

After treatment sera were diluted serially two fold with 0.01 M phosphate buffered saline, pH 7.2, using a microtiter system (Sever, 1962). Antibody titers are given as the reciprocals of the highest dilutions of the sera showing complete hemagglutination inhibition after incubation for one hour at room temperature. Four HA units of the following strains, A2/Aichi/2/68, A2/Kumamoto/1/67, and B/Tokyo/1/67, were used as antigens.

RESULTS

1. *Side reactions due to vaccination*

Neither epidemics of Hong Kong influenza, nor outbreaks of other respiratory diseases occurred during the period of vaccine administration. Systemic reactions such as fever, headache and fatigue were observed in less than 10% of the vaccinees on the day of vaccine administration. A febrile reactions of more than 38 C was observed in ca. 1% of the children.

As local reactions, 30 to 40% of the vaccinees complained of moderate local pain and few percent developed a red area of more than 40 mm in diameter at the site of injection. The results are summarized in Table 3.

2. *Antibody responses to A2 Hong Kong and B type influenza virus after vaccination*

All paired sera from the vaccinees were tested for antibody responses in the NT test using the antigen of the A2/Osaka/111/69 virus (A2 Hong Kong type) and in HI tests using the antigens of the A2/Aichi/2/68, A2/Kumamoto/1/67, and B/Tokyo/1/67 viruses. The results are shown in Table 4 and Fig. 1. About 4-fold increase in NT antibody titer and 8-fold increase in HI antibody titer were observed in response to A2 Hong Kong virus (Fig. 1 and Table 4). The ratio of sera showing a 4-fold rise or more after vaccination was 73.8% in the NT test and 83.7% in the HI test (Table 4). In the HI test the antibody response was more than in the NT test. This may be due to the high seronegative ratio, in other words, the low sensitivity of the test, for measurement of HI antibody.

There was about 2-fold increase in antibody

TABLE 3. *Side reactions due to vaccination*

Reactions			Occurrence of reactions in vaccinees					
			First vaccination			Second vaccination		
			Oct. 21	Oct. 22	Oct. 23	Nov. 6	Nov. 7	Nov. 8
Systemic Reactions	Fever	≥38 C	0.4%	0.2%	0 %	0.7%	0.2%	0 %
		≥37 C	5.7	0.8	0	0.7	0	0.2
		>37 C	93.9	99.0	100	98.6	99.8	99.8
	Headache	+	7.0	4.5	2.2	4.2	0.9	0.2
		−	93.0	95.5	97.8	95.8	99.1	99.8
	Fatigue	+	8.8	3.9	1.7	8.1	1.3	0.4
		−	91.2	96.1	98.3	91.9	98.7	99.6
Local Reactions	Pain	severe[a]	0.9	0	0	1.1	0.5	0.4
		moderate	42.3	13.7	3.6	34.7	15.5	6.2
		none	56.8	86.3	96.4	64.2	84.0	93.4
	Redness	≥40 mm	0.5	0	0	0.7	0	0
		<40 mm	5.7	3.6	1.1	1.1	0	0
		none	93.8	96.4	98.9	98.2	100	100

a; Children complained of difficulty in moving the arm which had received the injection.

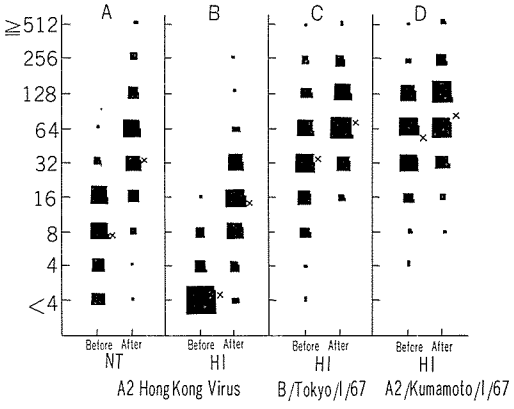


FIGURE 1. *Antibody Responses of Vaccinees to A2 Hong Kong Virus in the NT (A) and HI (B) Test and to B/Tokyo/1/67 (C) and to A2/Kumamoto/1/67 (D) in the HI Test.*
X: Geometric mean of titers.

titer to the B type influenza virus, B/Tokyo/1/67 (Fig. 1C). The response to the B type virus was less than that to the A2 Hong Kong virus (Fig. 1A, B and C). This may be partly because of the relatively high antibody level in the serum before vaccination and because the vaccine used contained less B virus than A2 Hong Kong virus antigen.

The antibody response to heterologous A2/Kumamoto/1/67 is also shown in Fig. 1D and Table 4. There was much less rise in antibody than with A2 Hong Kong virus because of the great antigenic shift of A2 type virus in 1968.

3. *Duration of antibody levels in vaccinees and children after natural infection*

The antibody levels of A2 Hong Kong virus in the groups of vaccinees and children with influenza after one year are shown in Fig. 2. The degree of decrease in the antibody titers was in the same in both groups. The geometric mean of the NT antibody titer decreased 1/2 (Fig. 2A and C) and that of the HI titer decreased to 1/3 (Fig. 2B and D) of the level soon after vaccination or infection.

As shown in Fig. 3, the decrease in antibody titers to B type virus were not great in either

TABLE 4. *Antibody response of vaccinees to several antigens*

Antibody Titer before Vaccintion	Vaccinees with 4-fold or more rise in antibody / Total vaccinees			
	A2/Hong Kong/68		B/Tokyo/1/67 HI-Ab. (%)	A2/Kumamoto/1/67 HI-Ab. (%)
	NT-Ab. (%)	HI-Ab. (%)		
<4	28/30 (93.4)	133/153 (87.0)	2/2 (100)	
4	22/25 (88.0)	19/21 (90.5)	1/1 (100)	1/2 (50.0)
8	39/51 (71.5)	8/16 (50.0)	15/18 (83.3)	0/3 (0)
16	36/59 (61.0)	0/1 (0)	18/35 (51.4)	7/17 (41.2)
32	4/8 (50.0)		15/68 (22.1)	10/57 (17.6)
64	1/1 (100)		2/42 (4.8)	5/62 (8.1)
128			0/18 (0)	1/46 (2.2)
256			0/7 (0)	0/4 (0)
≥512			0/1 (0)	0/1 (0)
Total	130/174 (73.8)	160/191 (83.7)	53/192 (22.6)	24/192 (12.5)

TABLE 5. *Comparison of antibody responses of vaccinees with those of children infected with A2 Hong Kong virus*

Subjects		Geometric mean of titers		*a/b*
		Log_2 NT-Ab.$=a$	Log_2 HI-Ab.$=b$	
Vaccinees	before	2.92	1.28	1.64
	after	5.06	3.83	1.23
Infected Children (A)	before	3.25	1.62	1.63
	after	8.06	6.86	1.20
Infected Children (B)	before	3.88	2.88	1.00
	after	9.13	7.83	1.30

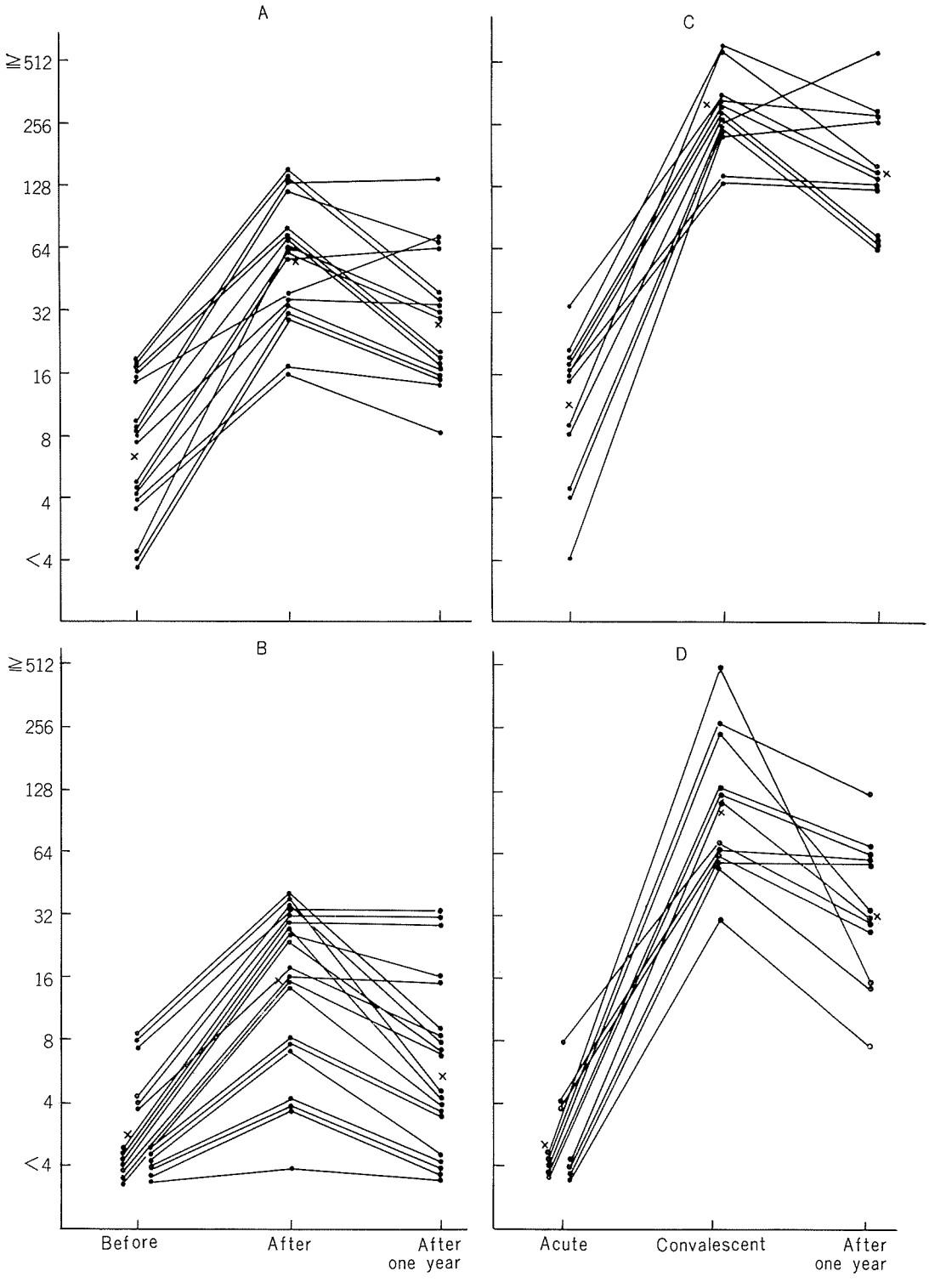
Children in groups A and B contracted A2 Hong Kong influenza during the epidemics of 1968 to 1969, and 1969 to 1970, respectively.

the vaccinees or children with influenza. In the case of heterologous A2 virus, the decrease in the antibody level of the children with influenza was less than 1/2 the geometric mean (Fig. 4B), whereas no decrease was observed in the vaccinees (Fig. 4A).

4. *Correlation between the NT antibody titer and the HI antibody titer*

The correlation between the NT antibody titer and the HI antibody titer is shown in Fig. 5 and Table 5. The NT antibody titers were consistently 2 to 3 fold higher than the HI antibody titers. This is in accordance with the results of Kan et al. (1970).

By the NT test and HI test 17.3% and 80.2 %, respectively, of the vaccinees were seronegative before vaccination. The antibody levels of vaccinees who were seronegative by the HI test before vaccination remained at 1 : 8 to 1:16 by the NT test, even after vaccination, as shown in Fig. 5A and B. This may be due to retention of high antibody levels from previous infection with A2 influenza virus.


A
B
C
D
≧512
256
128
64
32
16
8
4
<4
Before
After
After one year
Acute
Convalescent
After one year

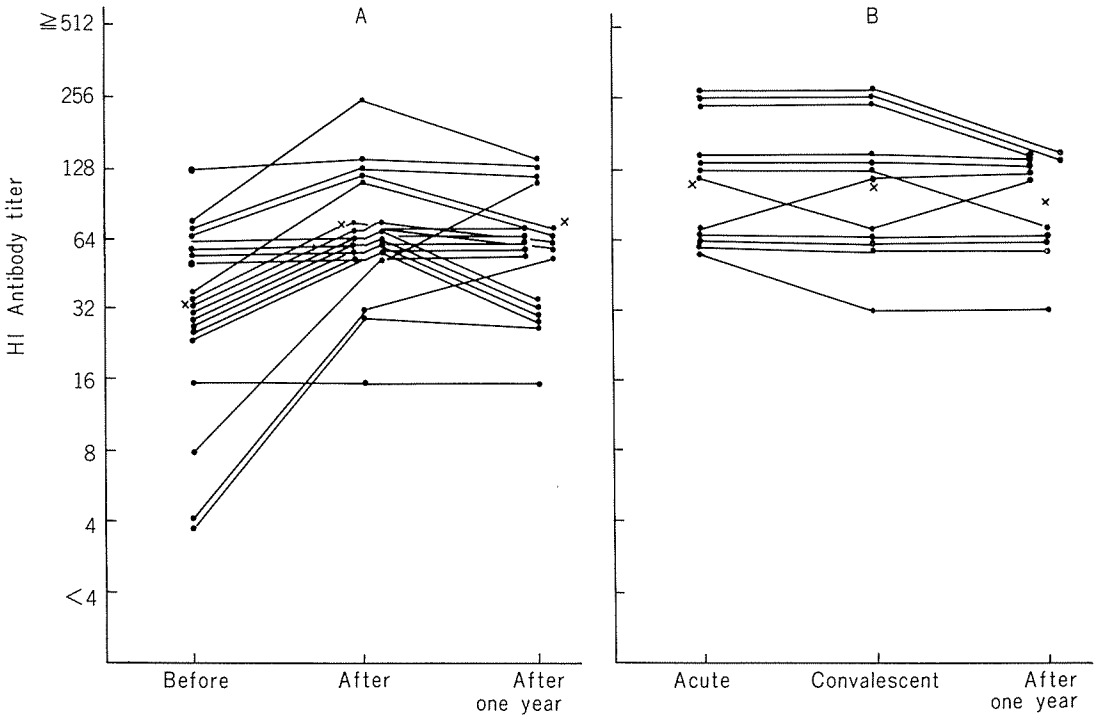


FIGURE 3. *Follow-up Data of the HI Antibody Responses to B/Tokyo/1/68, of Vaccinees (A) and Infected Children (B).*

X: Geometric mean of titers.

5. *Comparison of the antibody responses in vaccinees and children with Hong Kong influenza virus*

The accumulative percentages of the NT and HI antibody responses of vaccinees and of children with A2 Hong Kong influenza, during the epidemics of 1968 to 1969, are shown in Fig. 6.

The rise in the antibody titer in the sera of vaccinees in both the NT and HI test is indicated and the geometric means of the NT antibody titers and HI antibody titers are shown in Table 5.

◀

FIGURE 2. *Follow-up Data of Antibody Responses of Vaccinees in the NT Test (A) and HI Test (B), and of Infected Children in the NT Test (C) and HI Test, (D), to A2 Hong Kong Virus.*

X: Geometric mean of titers.

Fig. 6A, shows that when the vaccine used was administered by the routine method, about 80% of the vaccinees developed an NT antibody level of more than 1: 32. As this level is believed to the lower limit for protection against infection, about 80% of the vaccinees are unlikely to become infected by A2 Hong Kong influenza and about 55% of those who have an NT antibody level of 1: 64 may be protected against A2 Hong Kong influenza infection.

As shown in Table 5, the NT and HI antibody levels in infected children in group B during the acute period of infection during the winter of 1969 to 1970, were higher than in those of group A in the winter of 1968 to 1969. NT antibody levels of 1: 16 and of 1: 32 were observed in 58.7% and 17.6% of the patients, respectively. None of the 17

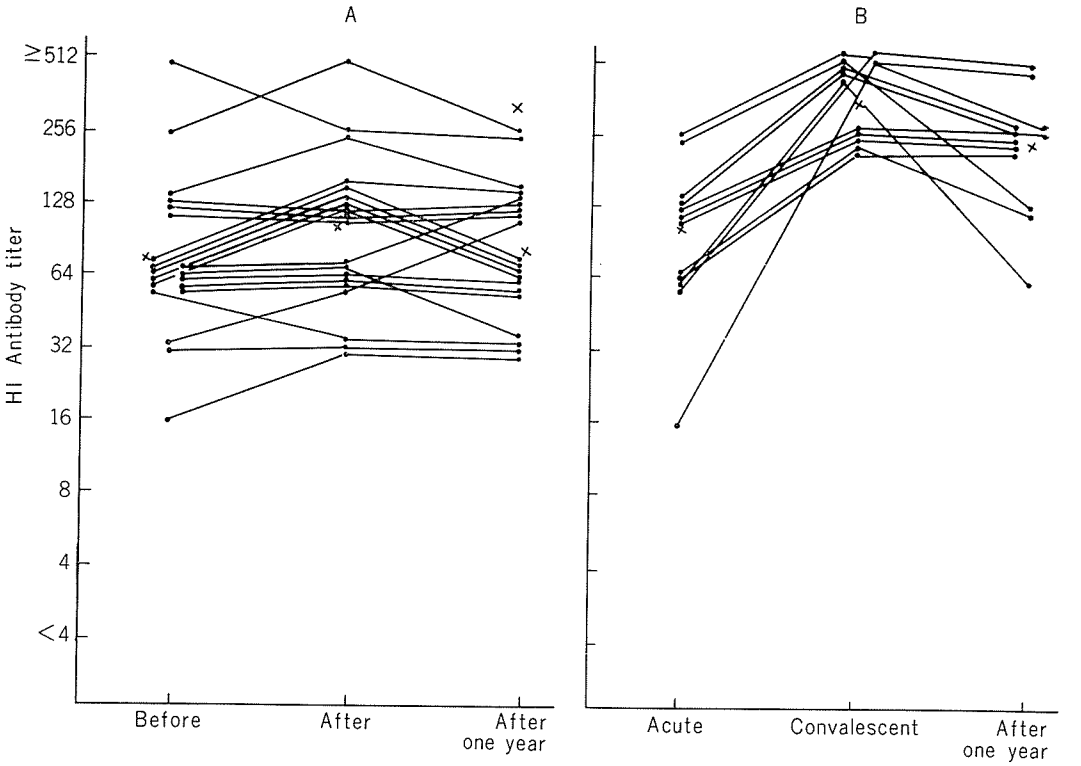


FIGURE 4. *Follow-up Data of the HI Antibody Responses to A2/Kumamoto/1/67, of Vaccinees (A) and Infected Children (B).*
X: Geometric mean of titers.

patients had an antibody level of more than 1: 64. These results indicate that individual antibody levels of 1: 64 in the NT test may be required for complete protection against A2 Hong Kong influenza.

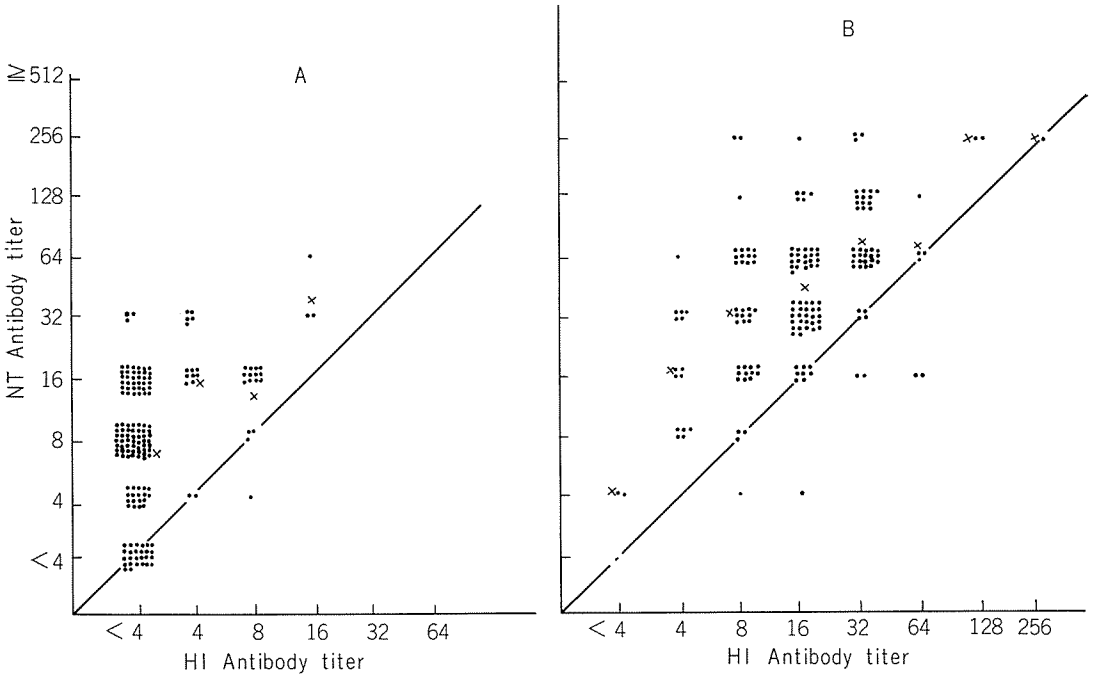
DISCUSSION

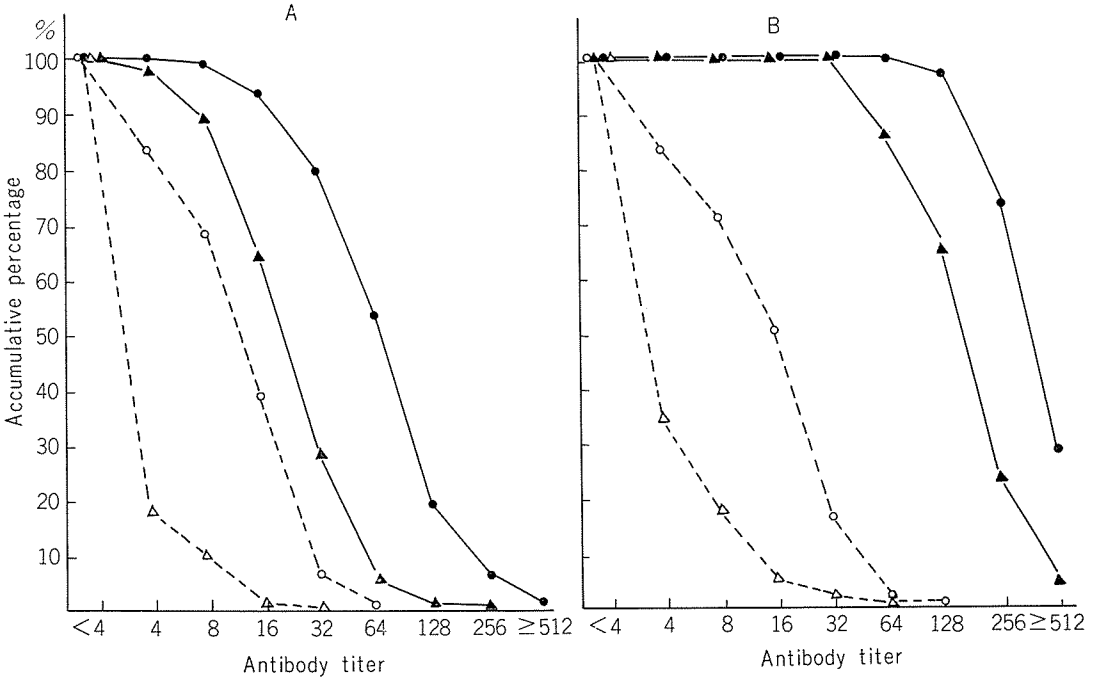
Mass administration of the inactivated influenza vaccine to school children is the best way to prevent epidemics of influenza because children of school age show the greatest incidence of influenza and are important in disseminating the virus. An epidemic of Hong Kong influenza was anticipated in the winter of 1968 to 1969 in Japan and a field trial was made of vaccine composed of A2 Hong Kong and B type influenza virus to school children in Osaka City.

More than 95% (ca. 540) of the children in the school were vaccinated and about 10% of the vaccinees showed slight systemic reactions on the day of vaccination. Eighty per cent of the vaccinees showed 4-fold rise or more in the NT antibody titer after vaccination. The antibody response was good, but the authors

FIGURE 5. *Relationship between NT Antibody Titer and HI Antibody Titer in Sera before (A) and after (B) Vaccination.*
X: Geometric mean titers.

FIGURE 6. *Antibody Responses of Vaccinees (A) and Infected Children (B) to Hong Kong Influenza, in Tests (before: ○------○, after: ●——●) and HI Tests (before: △------△, after: ▲——▲).*


A
B
NT Antibody titer
≧512
256
128
64
32
16
8
4
＜4
＜4
4
8
16
32
64
128
256
HI Antibody titer


A
B
%
Accumulative percentage
100
90
80
70
60
50
40
30
20
10
＜4
4
8
16
32
64
128
256
≧512
Antibody titer

are not satisfied with the results because side reactions occurred, although they were slight. The vaccine containing a total of 300 CCA units of virus per ml, should be improved, for use with children to eliminate side reactions. Zonal ultracentrifugation has been used in production of more purified vaccine of A2/Aichi/2/68 (Mostow et al., 1969). It was reported that administration of a low dose (300 CCA units/ml) of the highly purified vaccine caused systemic reactions in only a few percent of the vaccinees, while vaccination with a high dose (3000 CCA units/ml) caused side reactions in about 20 to 30% of the vaccinees. These findings suggest that side reactions are associated with the nature of the influenza virion and further modification of the pruified virus shoud be considered.

The NT antibody titers in vaccinees were generally 2 to 3-fold higher than the HI antibody titers and the NT antibody responses always corresponded well with the HI antibody titers.

Morris et al. (1966) reported that volunteers who had an NT antibody level of 1 : 320 were protected from infection with live A2 influenza virus in aerosol experiments and that some volunteers who retained NT antibody levels of more than 1 : 80 were not infected with A2 influenza virus (Alford et al., 1966). From our data on children infected with A2 Hong Kong influenza during the epidemics of 1968 to 1969 and 1969 to 1970 (Table 5 and Fig. 6B), an NT antibody level of 1 : 64 seems to be the lowest level for protection against A2 Hong Kong influenza. Vaccine containing 200 CCA units/ml of A2 Hong Kong influenza virus induced an excellent antibody response of this grade in our field trial. Sonoguchi (1969) reported the efficacy and a significant antibody response to the same lot of Hong Kong influenza vaccine which we used in a field trial about 3000 soldiers in Japan during the winter of 1968 to 1969.

Recently it has been implicated that IgA in the respiratory tract plays an improtant role in protection against influenza viruses (Alford et al., 1967; Waldman et al., 1967 and 1968; and Mann et al., 1968). Fulk et al. (1970) reported that among all the age groups vaccinated intranasally with vaccine of A2 Hong Kong influenza, children showed the greatest frequency of antibody response with increase in the mean antibody titers in both the serum and nasal secretions.

It is interesting that the decrease in the NT and HI antibody levels of vaccinees and patients with A2 Hong Kong influenza was almost the same after one year (Fig. 4). The antibody level induced by the vaccine, therefore, may be effective in preventing infection with A2 Hong Kong influenza even after one year, if IgG in the serum is the major factor in protection against influenza viruses. Our results and the above data are encouraging for application of inactivated influenza vaccine. The antibody titers to B/Tokyo/1/67 and to classical A2/Kumamoto/1/67 remained high one year after vaccination or infection. These results are probably due to repeated antigenic stimulations during previous epidemics of the classical A2 type and B type influenza viruses (Lief and Henle, 1960; Henle and Leif, 1963). A high antibody level to A2 Hong Kong influenza virus could also be induced in man by repeated stimulation with A2 subtype viruses which are antigenically similar to A2 Hong Kong influenza virus.

ACKNOWLEDGEMENTS

We wish to thank Miss Y. Takenaka and Miss T. Yoneda for their technical assistance.

REFERENCES

Alford, R. H., J. A. Kasel, P. J. Aerone and V. Knight. 1966. Human influenza resulting from aerosol inhalation. Proc. Soc. Exp. Biol. Med. 122: 800–804.

Alford, R. H., R. D. Rosser, W. T. Butler and J. A. Kasel. 1967. Neutralizing and hemagglutination-inhibition activity of nasal secretions following experimental human infection with A2 influenza virus. J. Immunol. 98: 724–731.

Fulk, R. V., D. S. Fedson, M. A. Huber, J. R. Fitzpatrick and J. A. Kasel. 1970. Antibody responses in serum and nasal secretions according to age of recipient and method of administration of A2/Hong Kong/68 inactivated influenza virus vaccine. J. Immunol. 104: 8–13.

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.

Kan, T., K. Koyama, K. Takaku, M. Takahashi and N. Kunita. 1970. Improvement of the method of plaque formation of influenza virus and its application to neutralization test. Biken J. In the press.

Lief, F. S. and W. Henle. 1960. Antigenic analysis of influenza viruses by complement fixation. VI. Implication of age distribution of antibodies to heterologous strains following infection and vaccination. J. Immunol. 85: 494–510.

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: 736–735.

Morris, J. A., J. A. Kasel, M. Saglam, V. Knight and F. A. Loda. 1966. Immunity to influenza as related to antibody levels. New Eng. J. Med. 274: 527–535.

Mostow, S. R., S. C. Schoenbaum, W. R. Dowdle, M. T. Coleman and H. S. Kaye. 1969. Studies with inactivated influenza vaccines purified by zonal centrifugation. (Adverse reactions and serological responses.) Bull. WHO. 41: 525–530.

Sonoguchi, T. 1969. Hemagglutination-inhibiting antibody response to and efficacy of inactivated Hong Kong influenza vaccine. Bull. WHO. 41: 517–523.

Waldman, R. H., J. J. Mann and J. A. Kasel. 1967. Immunoglobulin classes of serum neutralizing antibody formed in response to immunization with dead influenza virus vaccine. Proc. Soc. Exp. Biol. Med. 126: 888–891.

Waldmann, R. H., J. J. Mann and J. A. Kasel. 1968. Influenza virus neutralizing antibody in human respiratory secretions. J. Immunol. 100: 80–85.

Waldmann, R. H., J. A. Kasel, R. V. Fulk, Y. Togo, R. B. Hornick, G. G. Heiner, A. T. Dawkins and J. J. Mann. 1968. Influenza antibody in human respiratory secretions following subcutaneous or respiratory immunization with inactivated virus. Nature. 218: 594–595.

Waldman, R. H., J. J. Mann and P. A. Small. 1969. Immunization against influenza. J. Amer. Med. Ass. 207: 520–524.