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Serological evaluation of an influenza A virus cold-adapted reassortant live vaccine, CR-37 (H1N1), in Japanese adult volunteers

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SUMMARY

A cold-adapted influenza A virus, CR-37 (H1N1), derived from genetic reassortment between A/Ann Arbor/6/60 (H2N2) cold-adapted variant virus and A/California/10/78 (H1N1) wild-type virus, was tested in Japanese adult volunteers. The CR-37 live virus preparation induced only low-grade clinical reactions in volunteers for the first 3-4 days after inoculation. Two vaccinees who did not show any antibody changes became febrile (over 38·0 °C). Skin tests using the vaccine preparation and uninfected allantoic fluid were performed, and indicated that one of these two vaccinees was positive for the CR-37 vaccine preparation. A high proportion of the vaccinees whose sera had a haemagglutination-inhibition (HI) antibody titre against the vaccine strain of $\leqslant 64$ before inoculation, seroconverted in both HI and neuraminidase-inhibition (NAI) antibody titrations, and only a few seroconverted in the titration of antibody against type-specific internal antigens. The serological examinations against heterotypic H1N1 variants indicated that the cold-adapted live influenza virus vaccine could induce a broad spectrum of HI antibody reactivity and immunity of long duration.

INTRODUCTION

In recent years, considerable efforts have been made to develop live attenuated influenza virus vaccines as an alternative approach for the prevention of influenza (Chanock & Murphy, 1980). Vaccination by live vaccine entails subjecting susceptible individuals to infection with the attenuated virus rather than intramuscular injection of inactivated viral antigen. The master strain, an influenza A/Ann Arbor/6/60 (H2N2) cold-adapted (*ca*) variant, provided two phenotypic genetic markers in the attenuation, namely cold-adaptation and temperature sensitivity (Maassab, 1967; Maassab *et al.* 1969). A series of reassortants between

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the *ca* master strain and recent isolates (H1N1 or H3N2 wild-type strains) were administered intranasally to volunteers in the U.S.A. and in Europe, and the results indicated that satisfactory attenuation of the vaccine virus had been achieved (Maassab *et al.* 1977; Moritz *et al.* 1980; Murphy *et al.* 1979, 1980, 1981, 1982; Reeve *et al.* 1980; Wright *et al.* 1982).

The study described in this communication was initiated to evaluate a reassortant of the A/Ann Arbor/6/60 (H2N2) *ca* virus and an H1N1 isolate. The *ca* reassortant vaccine strain was designated as CR-37, and possessed both the haemagglutinin (HA) and the neuraminidase (NA) of influenza A/California/10/78 (H1N1). Our results demonstrate that the *ca* live vaccine preparation has two main advantages over traditional inactivated influenza virus vaccine, regarding the duration and breadth of immunity induced.

MATERIALS AND METHODS

Vaccine

The vaccine strain employed in this study was CR-37 (H1N1) which is a genetic reassortant between *ca* master A/Ann Arbor/6/60 (H2N2) and A/California/10/78 (H1N1). It is well established that only the genes coding for the envelope antigens, HA and NA, were derived from the wild-type A/California/10/78 (H1N1) (Maassab *et al.* 1982). An experimental vaccine was prepared in specific pathogen-free (SPF), fertile chicken eggs inoculated by the allantoic route. As placebo material, allantoic fluid from uninfected SPF fertile chicken eggs was used following incubation procedures used for vaccine preparation. These vaccine preparations were kindly donated by Dr John R. LaMontagne, the National Institute of Allergy and Infectious Diseases, MD, U.S.A. through Flow Laboratories Inc., VG, U.S.A.. The vaccine preparation contained $10^{7\cdot0}$ TCID50 per ml of CR-37 virus, determined in primary chick kidney (PCK) cell cultures.

Clinical study

Study participants were selected from healthy student volunteers of Tohoku University College of Medical Technology and Care, Sendai, Japan. Contraindications to inclusion in the study were known allergy to egg protein or chicken feathers, acute or chronic illness, and pregnancy. The purpose and possible risks of the study were explained in detail to the volunteers, who signed informed consent forms. A total of 27 volunteers from 20 to 35 years old were employed in the study. Five out of the 27 participants were randomly selected for placebo inoculation, and they were unaware of which preparation they received. Virus or placebo was inoculated by intranasal instillation of 1·0 ml of undiluted materials (0·5 ml per nostril) while the volunteers were in a supine position.

Daily, the volunteers recorded the presence or absence of respiratory symptoms (sneezing, nasal discharge, sore throat, cough or sputum) and subjective systemic symptoms (headache, joint pain, myalgia or lumbago). All the symptoms observed were graded from 0 to 3+ by the vaccinees themselves. Body temperatures were measured orally twice daily, in the morning and evening. Body temperatures over 38·0 °C were regarded as indicative of a febrile reaction.

Laboratory tests

Serum specimens were collected before vaccination, and then once a week for the first month after inoculation. In the successive months, venous blood samples were obtained once a month for approximately one year.

The standard haemagglutination-inhibition (HI) test was employed using the following antigens: A/California/10/78 (H1N1), A/Fukushima/103/78 (H1N1), A/USSR/92/77 (H1N1), A/FM/1/47 (H1N1), A/New Jersey/8/76 (Hsw1N1). Anti-neuraminidase antibody titres were determined by the neuraminidase-inhibition (NAI) technique using the antigen of A/equine/Prague/1/56 (Heql)-A/USSR/90/77 (N1), by a chemical titration with fetuin substate (Palmer *et al.* 1975; Yamane *et al.* 1981). For the titration of antibody against the type-specific soluble ('s') antigens of influenza A and B viruses, the single-radial-complement-fixation (SRCF) test was employed. It has been shown that the SRCF test only detects antibody against nucleoprotein (NP) (Yamane, Yuki & Nakamura, 1983).

Virus isolation was performed by inoculating pharyngeal swabs into Madin-Darby canine kidney (MDCK) cell cultures in the presence of 5 μg/ml of trypsin, followed by incubation at 33 °C. The pharyngeal swabs were collected on days 1, 2, 3, 5, 7 and 10 after vaccination. Temperature sensitivity and *ca* phenotypes of the isolates were determined in MDCK cell cultures (Odagiri, DeBorde & Maassab, 1982).

Skin test to vaccine preparation

The selected vaccinees who became febrile were given vaccine preparation in skin tests. One hundred microlitres of active CR-37 vaccine preparation and uninfected allantoic fluid, undiluted and diluted 1 in 100, were administered intradermally along with phosphate buffer saline (PBS) as control. The diameter of the erythema which appeared was measured 24 and 48 h after injection.

RESULTS

Early (during the first month) serological responses after vaccination

(*1*) *Anti-HA antibody responses.* Fig. 1 shows the HI antibody titre changes against A/California/10/78 (H1N1) in sera collected from the vaccinees who received active CR-37 (H1N1) vaccine preparation, for all vaccinees and for HI antibody seronegatives (< 16). Out of 22 vaccinees, 13 persons (59%) showed significant HI antibody rises during the first month after inoculation. The percentage seroconversion for each pre-existing HI antibody level was three out of five (60%) for those with a titre of 64, three out of three (100%) for those with a titre of 16, and seven out of nine (78%) of seronegatives. Four subjects whose sera possessed HI antibody at a titre of 128 before vaccination did not show any change in antibody titre after vaccination. Significant HI antibody rises were observed in four persons in the first week, eight in the second, and one in the fourth week after vaccination. The cumulative HI antibody titre distribution against A/California/10/78 (H1N1) revealed that, before vaccination, only 18% (4/22) of the vaccinees possessed an HI antibody titre of 128 against the vaccine strain, and then the number of subjects with an HI titre of $\geqslant 128$ gradually increased during

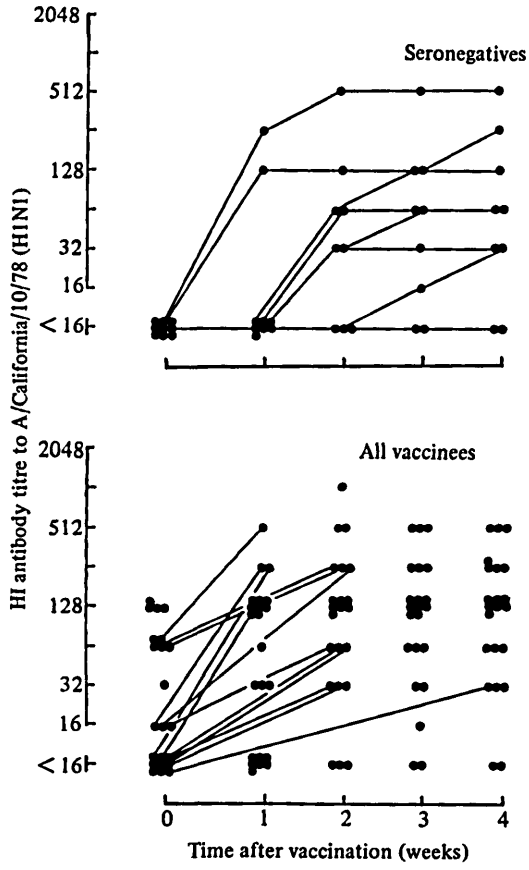

Fig. 1. HI antibody responses against A/California/10/78 (H1N1). The upper figure shows the results for seronegatives (HI titre < 16 before vaccination), and the lower the results of all the vaccinees. In the lower figure lines are drawn between pre-vaccination sera and the first post-vaccination serum to show a significant antibody rise.

the weeks after vaccination. The maximum percentage of persons with such HI titres was 64% (14/22) and was obtained in the third week after inoculation (Fig. 2). The geometric mean titre (gmt) of HI antibody among vaccinees was 28·2 before vaccination, and 116·5 four weeks after vaccination. Also, the gmt of seronegatives rose to 55 (data not shown).

The distribution of cumulative HI antibody titres against various H1N1 variants, A/FM/1/47 (H1N1), A/Fukushima/103/78 (H1N1) and A/USSR/92/77 (H1N1) are shown in Fig. 2, as well as those against the homologous A/California/10/78 (H1N1). As can be seen, the vaccine induced antibody rises against all the H1N1 strains tested. The percentage of persons whose sera had HI antibody titres $\geqslant$ 128 increased by 30–50% for each HA antigen. The same trend was also observed with the seronegatives (data not shown). However, no vaccinee seroconverted against Hswl antigen of A/New Jersey/8/76 (Hsw1N1).

(2) *Anti-NA antibody response.* Significant NAI antibody rises against A/USSR/90/77 (N1) were observed in eight (36%) of the 22 vaccinees (Fig. 3).

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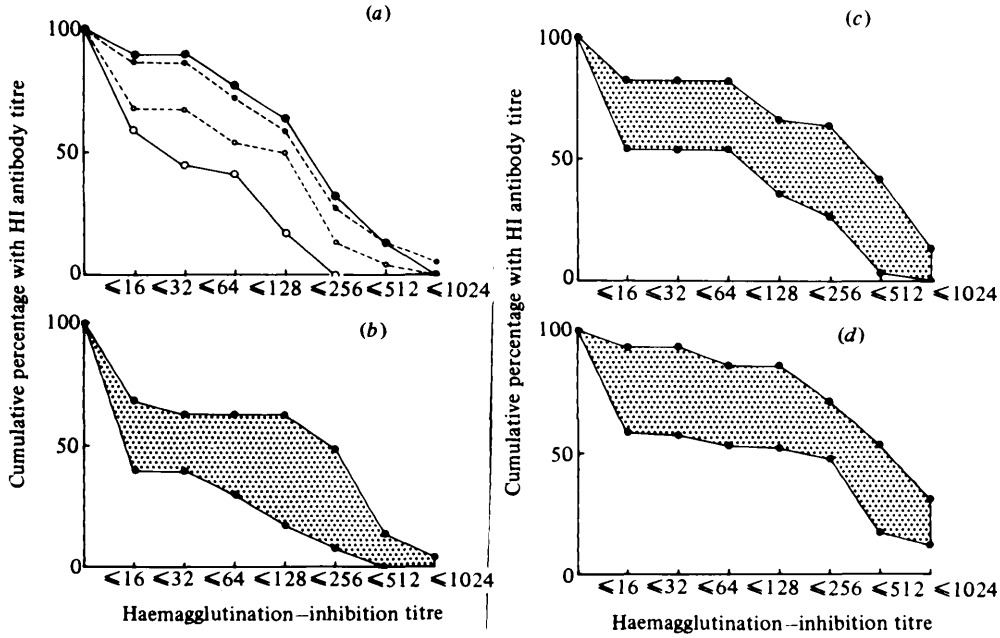

Fig. 2. Distributions of cumulative HI antibody titres against various H1N1 variants before and after vaccination. (*a*) The distribution of HI antibody against A/California/10/78 (H1N1); before vaccination (○——○), 1 week (○- - - -○), 2 weeks (●- - - -●) and 4 weeks (●——●) after immunization. (*b*) The distribution of HI antibody against A/USSR/92/77 (H1N1) before and 4 weeks after vaccination. (*c*) Identical to (*b*), but against A/FM/1/47 (H1N1). (*d*) Identical to (*b*), but against A/Fukushima/103/78 (H1N1).

Of these eight persons, six showed antibody rises in both HI and NAI tests, but the remaining two showed a significant antibody rise only against the NA (N1) antigen. Fig. 3 shows that the significant antibody rises against NA antigen were observed earlier than those against HA antigen, that is, six out of eight subjects showed significant NAI antibody rises in the first week after vaccination. However, among the individuals whose sera were negative in NAI titration (titre < 2) before vaccination, only five (45%) of 11 vaccinees exhibited seroconversion. For the group as a whole the gmt was 4 and 13 before and four weeks after vaccination, respectively.

(*3*) *Antibody response against type-specific 's' antigen.* Fig. 4 shows the antibody response against 's' antigen of influenza A virus tested by single-radial-complement-fixation (SRCF) test. As shown in the figure, only four vaccinees showed significant antibody rises against the type-specific 's' antigen in the SRCF test. By four weeks after vaccination, most of the antibody levels were elevated, but almost all the rises were not significant.

Clinical response to vaccine inoculation

Two vaccinees were febrile (over 38·0 °C), one on the day of inoculation and the other on the 2nd, 3rd and 4th days after inoculation. However, both vaccinees were shown to possess HI antibody titres of 128 against the vaccine strain before

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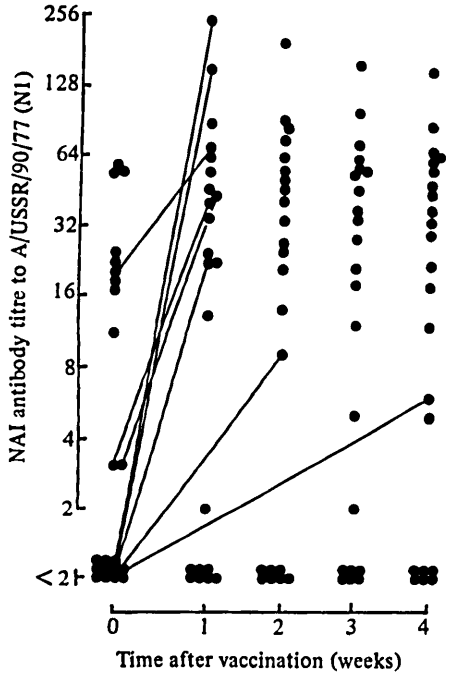

Fig. 3. Anti-neuraminidase antibody responses against A/USSR/90/77 (N1) in all vaccinees. Lines join pre-vaccination sera with the first post-vaccination serum to exhibit a significant antibody rise.

vaccination, and the paired sera obtained did not show any antibody changes against HA, NA or type-specific 's' antigens. Several viral antigens, including influenza B virus, paramyxovirus types 1–4, adenovirus, respiratory syncytial virus, mumps, rubella, measles viruses, and *Mycoplasma pneumoniae* were serologically tested, but all results were negative in the paired sera. One of the two cases was skin-test positive (Table 1). A small area of erythema appeared where normal uninfected allantoic fluid was tested. However, a more extensive positive reaction was observed against the CR-37 vaccine preparation, maximally at 24 h after administration. It is likely that the case was allergic to both allantoic fluid and virion components; however, the positive skin test was mainly attributable to a reaction with virion components.

Twenty-seven volunteers including those receiving placebo inoculation complained of mild upper respiratory symptoms such as sneezing, sore throat, or nasal obstruction, and some of these complained of systemic reactions, such as headache or myalgia. Fig. 5 summarizes the subjective symptoms as a score index for each day after inoculation among the active CR-37 vaccinees. As can be seen, almost all the symptoms were experienced on the second day after vaccination, and then gradually improved. However, we could not find any significant relationship between the grade of clinical reactions and serological responses in each individual (data not shown).

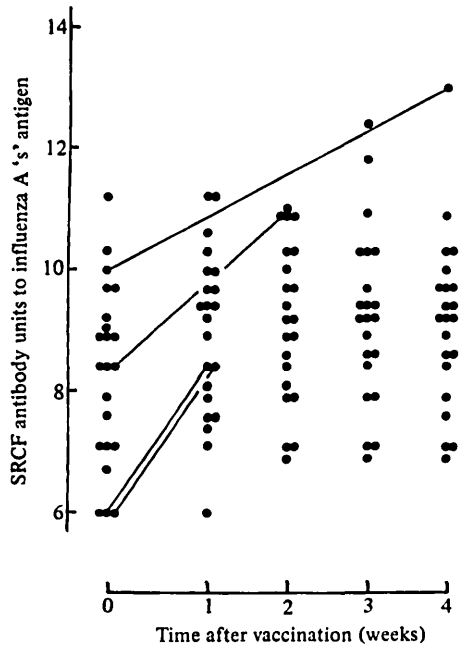

Fig. 4. Antibody responses against type-specific 'soluble' antigen of influenza A virus estimated by the single-radial-complement-fixation (SRCF) test. Lines join pre-vaccination sera with the first post-vaccination serum to exhibit a significant antibody rise.

Table 1. *The results of intradermal skin test of a vaccinee who became febrile after vaccination with* ca *virus*

	Diameter of area of erythema (mm)	
Antigen	24 h after injection	48 h after injection
CR-37 vaccine preparation		
Undiluted	32·6	24·5
Diluted 10^{-2}	16·7	10·6
Uninfected allantoic fluid		
Undiluted	10·8	6·5
Diluted 10^{-2}	4·4	3·0
Phosphate buffer saline (PBS)	None	None

Virus recovery from pharyngeal swabs and its characterization

Of the 22 vaccinees, only one was positive for virus isolation from a pharyngeal swab taken two days after vaccination. However, this person did not show any significant serological response. The isolate was shown to possess the antigenic configuration of H1N1, temperature sensitivity, no growth at 39 °C, and cold-adaptation (2×10^4 plaque forming units (p.f.u.) per ml at 25 °C and 4×10^4 p.f.u. per ml at 33 °C).

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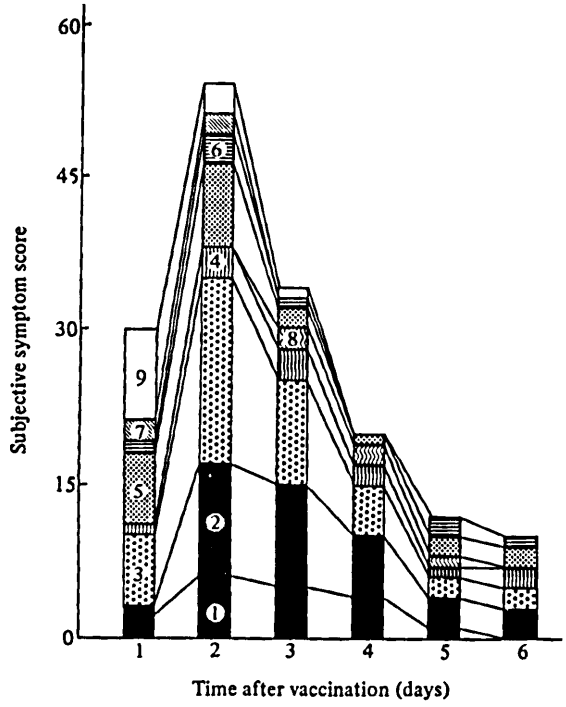

Fig. 5. Subjective reactions experienced by vaccinees after CR-37 live vaccination. Symptom grades estimated by the vaccinees themselves are summarized in the symptom scores. 1, sneezing; 2, nasal obstruction; 3, sore throat; 4, cough; 5, headache; 6, lumbago; 7, myalgia; 8, sputum; 9, others.

One year observation of serum antibody level after vaccination

The 22 vaccinees were serologically examined during the following winter of 1982–3. Table 2 indicates the gmt of HI antibodies against four different H1N1 variants and NAI antibody against A/USSR/90/77 (N1), for all 22 vaccinees and those 15 who seroconverted. As mentioned above, the gmt of HI antibodies against various H1N1 variants rose and reached a maximum 3–4 weeks after vaccination, and then gradually decreased in parallel fashion. However, 50–70% of the peak antibody levels in all vaccinees and 38–60% of those who seroconverted were maintained for over eleven months after vaccination. Characteristically the gmt of HI antibodies against heterologous H1N1 variants decreased more slowly than the homologous HI antibody against A/California/10/78 (H1N1) strain in all vaccinees including those who seroconverted. The gmt of NAI antibody levels peaked earlier than that of HI antibodies, and decreased in the same manner as HI antibodies. HI antibodies against A/New Jersey/8/76 (Hsw1N1) were observed only in those vaccinees who received the whole inactivated vaccination of A/New Jersey/8/76 (Hsw1N1) in 1977.

Table 2. *Geometric mean titres (gmt) of HI and NAI antibodies against various H1N1 influenza variants over period of one year*

	Gmt of sera collected the stated number of months after vaccination											
	0	1	2	3	4	5	6	7	8	9	10	11
HI antibody titres to A/California/10/78 (H1N1)												
Total vaccinees ($N = 22$)	28·2	116·5	109·3	90·5	82·3	82·3	77·3	74·9	70·3	64·0	64·0	60·0
Seroconverts ($N = 15$)	20·2	154·0	134·0	101·6	92·6	92·6	77·0	77·0	73·5	67·0	67·0	58·4
A/USSR/92/77 (H1N1)												
Total vaccinees	21·9	77·3	66·0	64·0	64·0	62·0	60·1	58·2	56·4	58·2	53·0	53·0
Seroconverts	15·2	92·5	76·8	73·3	73·3	69·9	67·1	63·8	61·0	63·8	55·7	55·7
A/FM/1/47 (H1N1)												
Total vaccinees	39·9	159·6	159·6	159·6	149·8	145·2	132·1	128·0	124·0	120·2	124·0	112·8
Seroconverts	26·7	185·5	177·0	177·0	177·0	153·9	134·0	134·0	127·8	116·9	134·0	111·6
A/Fukushima/103/78 (H1N1)												
Total vaccinees	36·3	99·5	87·7	87·7	82·3	77·3	77·3	74·9	72·6	66·0	68·2	70·3
Seroconverts	25·4	101·6	84·4	84·4	73·5	70·2	67·0	67·0	64·0	58·4	61·1	61·1
NAI antibody titres to A/USSR/90/77 (N1)												
Total vaccinees	4·1	12·8	11·7	11·0	10·6	10·2	10·1	9·2	8·5	8·2	8·3	8·8
Seroconverts	3·2	15·9	14·5	13·5	12·5	11·9	11·9	10·0	9·4	9·1	9·7	9·7

Seronegatives, < 16 in HI and < 2 in NAI tests, were calculated as 8 and 1, respectively. Seroconverts were subjects whose sera showed significant antibody rises in either HI or NAI tests, or both.

DISCUSSION

In a previous publication, we concluded that the present split-vaccine of influenza virus induces a limited spectrum of HI antibody reactivity against heterologous variants, especially in persons who have not experienced natural exposure to the virus, and that it is very difficult to achieve good immunity against NA antigen (Yamane *et al.* 1981). Since then, our main efforts have been focused upon obtaining sufficient levels of immunity against influenza virus in unprimed individuals. The study described in this communication evaluates a live vaccine of influenza A virus as an alternative approach for the prevention of influenza.

Genetic stability and safety are the minimal criteria necessary for clinically evaluating live vaccine. As for the side effects of *ca*-live vaccine, almost all the vaccinees complained of mild upper respiratory symptoms including nasal discharge, nasal obstruction, or sneezing. Some of these would be regarded as non-specific reactions due to nasal instillation of materials rather than due to infection, since these symptoms were also observed in the subjects who received placebo materials. Some vaccinees complained of systemic symptoms, such as headache, myalgia or lumbago. Since the degree of these symptoms is likely to depend on the personality of the individual, it is impossible to estimate objectively these symptoms in comparison with present intramuscular vaccination. Consequently, it now appears necessary to determine to what degree these symptoms will be acceptable to vaccinees on a large scale. The two vaccinees who did not become infected with the CR-37 virus became febrile in this study. One of the two was positive in an intradermal skin test using the vaccine preparation. The results indicate that the subject was probably allergic to virion-associated materials as well as to egg protein. Upon further examination, the basophilic leucocytes collected from this subject showed high reactivity in histamine release *in vitro* when the vaccine preparation was added (personal communication from Dr S. Ida, the First Department of Internal Medicine, Tohoku University School of Medicine, Sendai, Japan).

Virus was isolated from a single individual on only one occasion. Perhaps nasal washing would have afforded us better results. Unfortunately, therefore, we could not definitely estimate the genetic stability of CR-37 virus in the adult volunteers of this study. However, genetic instability of the *ts* phenotype has not been observed in humans for any of the *ca* reassortants so far tested (Murphy *et al.* 1979).

The immune responses induced by *ca*-live virus vaccine differ in several aspects from those observed with inactivated influenza virus vaccine. The serum HI antibody response to *ca*-live vaccine continued longer than that observed in response to inactivated vaccine, after which serum antibody levels significantly decline within six months of vaccination (Lerman, Wright & Patil, 1980). Also, a single dose of *ca*-live vaccine, approximately 10^7 TCID50 is sufficient to induce immunity against the virus, whereas the present split-product, inactivated vaccine usually requires two doses of concentrated virus. In addition, recent studies demonstrated that *ca*-live vaccine induces development of lymphocyte responsiveness as measured by lymphocyte transformation (Lazar, Okabe & Wright, 1980), and that local respiratory tract immunity is also stimulated (Murphy *et al.* 1982).

The breadth of serum HI antibody reactivity induced by *ca*-live vaccine is

characteristic. Although none of the vaccinees acquired HI reactivity against Hsw1 antigen, the serum HI antibody was also reactive against various heterotypic H1N1 variants, even in those subjects who were seronegative before immunisation. Additionally, heterotypic reactivity was maintained for almost one year. Our observation was reinforced by similar results with *ca*-Hong Kong reassortants, which indicated that *ca*-live vaccine protects against challenge from viruses related but not identical to the strain of H3N2 viruses (Wright *et al.* 1977).

It should not be concluded from our data, however, that *ca*-live vaccine can provide protective immunity to wild-type virus, since we could not evaluate this protection during natural exposure to the related virus. However, *ca*-live vaccine appears to be a suitable candidate for large-scale clinical trials in the near future.

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