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# CHARACTERIZATION AND CLINICAL EVALUATION OF LIVE INFLUENZA A VACCINE PREPARED FROM A RECOMBINANT OF THE A/USSR/92/77 ($H_1N_1$) AND THE COLD-ADAPTED A/ANN ARBOR/6/60 ($H_2N_2$) STRAINS

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Live influenza vaccine was prepared after genetic recombination of the A/USSR/92/77 ($H_1N_1$) strain with the cold-adapted A/Ann Arbor/6/60 ($H_2N_2$) strain. The recombinant contains the genes coding for the HA and NA proteins from the A/USSR/92/77 ($H_1N_1$) strain and the genes coding for the $P_1$, $P_2$, $P_3$, NP, M and NS proteins from the A/Ann Arbor/6/60 ($H_2N_2$) strain. To assess the properties of this vaccine, it was administered under double-blind conditions to 14 healthy volunteers, while another 14 healthy volunteers received placebo.

The vaccine virus appeared to be sufficiently attenuated. No febrile reactions were observed The vaccinees showed an increase in mean serum haemagglutination-inhibiting antibody level from 19 to 73 after two vaccinations From nasal swabs and antibody responses, it was concluded that the vaccine virus showed no transmission to the placebo group under conditions of close contact. Also, the vaccine virus was found to be genetically stable.

It is concluded that this live influenza virus vaccine meets the requirements for safe use in humans. However, several problems still exist which may impede a general use of live influenza vaccines

live influenza vaccine  cold-adapted recombinant  genotyping  reactogenicity  antibody response

## INTRODUCTION

Attenuated live influenza virus vaccines could mean an improvement over current inactivated influenza vaccines since they simulate more closely a natural infection. It is hypothesized that the protection they confer will be more efficient and longer lasting [16]. One approach for rapidly obtaining attenuated virus for vaccine production with a new antigenic variant is by its recombination with a well-characterized, genetically stable, attenuated donor strain. Characterization and genotyping of the recombinant is essential [21]. Ideally, the recombinant should contain the genes coding for the haemagglutinin and neuraminidase proteins from the wild-type parent and the other six genes from the attenuated donor strain. The genes coding for the surface proteins are presumed not to contribute to the virulence, although conflicting evidence exists [19, 23].

In our programme to investigate the potential usefulness of live influenza vaccines,

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we have used as a donor strain the cold-adapted A/Ann Arbor/6/60 strain [14]. This strain has been shown to be attenuated in man [13]. Earlier studies with live vaccines prepared by genetic recombination of this A/Ann Arbor/6/60 strain, with relevant antigenic variants of the $H_3N_2$ subtype, have shown that such cold-adapted recombinant vaccines can be immunogenic and without side effects [6,10,17], although the recombinants used were not always fully characterized.

For the study reported here, we prepared and characterized a recombinant of the cold-adapted A/Ann Arbor/6/60 strain with the A/USSR/92/77 strain belonging to the recurring $H_1N_1$ subtype. After selection of the appropriate recombinant, the vaccine was tested clinically in May, 1979, in young adult volunteers in a double-blind placebo-controlled study.

## MATERIALS AND METHODS

### *Viruses*

Wild-type virus A/USSR/92/77 ($H_1N_1$) was received from Sandoz Research Institute (Dr. P. Reeve). The cold-adapted A/Ann Arbor/6/60 ($H_2N_2$) donor strain, received from Dr. H.F. Maassab, has been described previously [14].

### *Preparation of recombinant virus*

Recombinant virus was obtained by double infection of primary chick embryo kidney tissue cultures with wild-type A/USSR/92/77 ($H_1N_1$) and cold-adapted A/Ann Arbor/6/60 ($H_2N_2$) viruses. Selection of antigenically appropriate recombinants was accomplished by two passages in the presence of mouse antiserum against A/Ann Arbor/6/60. This was followed by two passages in medium without antiserum. The virus was then cloned three times by plaque isolation. The whole recombination procedure was performed at 25°C [15]. Seed virus for vaccine production was obtained by incubation of the recombinant in specific pathogen-free (SPF) eggs (3 days, 33°C).

### *RNA and protein electrophoresis*

Identification of the genetic constellation of the recombinant was done by polyacrylamide gel-electrophoresis, comparing the mobilities of viral RNAs and proteins of the recombinant with those of the parents. The purification of the virus and isolation of the RNA followed standard techniques [22], without the addition of yeast RNA. The RNA was labelled with [$^3$H]uridine (75 $\mu$Ci/ml) during replication in Madin–Darby canine kidney (MDCK) cells.

The RNA was dissolved in 100 $\mu$l deionized formamide and denatured by heating at 100°C for 45 s followed by cooling in ice. Slab gels (18 × 14 cm, 2.4% acrylamide and 0.14% methylene-bis-acrylamide) were prepared according to Palese and Schulman [20].

Electrophoresis was carried out at 25°C, 19 h 70 V. Detection of tritium-labelled RNA was performed as described by Bonner and Laskey [3] on Kodak XR X-ray films. Protein electrophoresis was performed according to Inglis et al. [11].

*Hamster studies*

Compared to wild-type virus, the recombinant virus must show a restriction of replication in the lungs of hamsters. The procedure used was essentially as described by Spring et al. [26]. The hamsters were sacrificed after 48 h. The viral titres of the lung and nasal tissue suspensions, expressed as 50% egg infective doses ($EID_{50}$), were estimated by the method of Reed and Muench [4].

*Vaccine and placebo*

After we obtained seed virus from an appropriate recombinant (DU 5 clone 15), vaccine was produced in larger quantities by Sandoz Research Institute, Vienna. Vaccine was prepared by inoculation of the seed virus in SPF eggs. After incubation for 73 h at 33°C, vaccine was harvested and centrifuged. It was stored at -70°C. Production, control and application of this batch of live influenza vaccine for this clinical study was approved by the Dutch Health Authorities. The vaccine yielded $10^{9.0 \pm 0.4}$ $EID_{50}$/ml at delivery and was shown to be free of adventitious agents Just prior to use, it was thawed and diluted 15-fold with Eagle's minimal essential medium to obtain $10^{7.8}$ $EID_{50}$/ml. The dose actually administered was confirmed by egg infectivity titrations of the dilution and was found to be $10^{7.4 \pm 0.4}$ $EID_{50}$/ml. The placebo consisted of the diluent only. Each dose of vaccine or placebo consisted of 0.5 ml fluid, administered as a coarse spray into the nostrils (0.25 ml in each) of the volunteers.

*Study population*

24 male and four female healthy volunteers (students) between 19 and 28 years of age (mean age 22.5 years) participated after passing a thorough medical examination. Pregnancy and known sensitivity to egg-proteins were among the exclusion criteria. The participants had not been vaccinated previously with an A/USSR/92/77 ($H_1N_1$) containing vaccine. Two-thirds of the population can be regarded as unprimed in view of their age and pre-vaccination antibody titres. Informed consent was obtained from all study participants. The vaccine and placebo treatment groups (14 volunteers in each group) were comparable with regard to age, sex, mean pre-vaccination antibody titres and number of persons with an undetectable pre-vaccination antibody titre. The vaccine group contained eight volunteers with an undetectable pre-vaccination antibody titre and the placebo group nine.

*Study design*

On admission to the study, an 8 ml blood sample was taken and a nasal swab collected from each volunteer (day 0). Directly thereafter, one dose of vaccine or placebo was administered in a double-blind fashion and according to a randomization list, using a mechanical device which reproducibly delivers a coarse spray. From that point on, all participants remained in a Dutch holiday resort, isolated from outside contact until no more virus shedding was to be expected (10 days), to prevent a possible transmission to the community. The participants lived those 10 days in seven bungalows situated closely to each other, where they played games, ate, slept, etc. Contact between the bungalows was encouraged and each bungalow contained both vaccine- and placebo-treated volunteers to ensure optimal contact between the two treatment groups.

Just before vaccination (day 0) and on days 1, 2, 3, 4 and 5 following vaccination, each volunteer was medically examined and his/her body temperature measured sublingually twice daily (morning and evening). Neither the volunteers nor the investigator performing all the medical examinations knew which treatment any individual had received until the end of the study.

Apart from the nasal swab taken on day 0, just prior to vaccination, nasal swabs were also collected on 1, 2, 3, 5, 7 and 9 days following the first vaccination, in order to detect any virus shedding. Each nasal swab was transported in 2 ml Eagle's MEM. Four weeks after the first vaccination, a second blood sample was obtained from each volunteer. Three days later, all volunteers received a second dose of vaccine or placebo. Each volunteer received the same treatment as during the first vaccination session. No clinical measurements were done after this second vaccination, nor were nasal swabs taken. The second vaccination was not carried out under closed trial conditions, as no virus transmission was observed after the first vaccination (see Results). Four weeks after the second vaccination, a third blood sample was taken from all volunteers.

*Serological techniques*

Non-specific inhibitors of haemagglutination were destroyed with cholera filtrate (Philips-Duphar B.V., Amsterdam). Haemagglutination-inhibiting (HI) antibodies were titrated in Microtitre® plates (Flow Laboratories Ltd., U.K.) using a standard technique [27]. Four haemagglutinating units of allantoic fluid, containing A/USSR/92/77, were allowed to react with 2 volumes of doubling dilutions of treated serum for at least 45 min at room temperature, before the addition of 1.0% chicken erythrocytes.

Titres are expressed as the reciprocal of the final serum dilution, causing 50% inhibition of haemagglutination. The titre assigned to each sample was the geometric average of two determinations. For calculation purposes, an undetectable antibody titre ($\leqslant 17$) was assigned a titre of 8.

Neuraminidase-inhibiting (NI) antibodies were determined according to Aymard-Henry et al. [1]. The NI-titre against A/USSR/92/77 assigned to each sample is expressed

as the dilution of serum giving 50% inhibition of neuraminidase activity. For calculation purposes, titres $< 3$ are expressed as 1. The titrations of the first, second and third blood sample of each subject were carried out in parallel.

## RESULTS

### *Cold-adapted recombinants*

Cold-adapted recombinants were produced by double infection of cells with cold-adapted A/Ann Arbor/6/60 virus and a wild-type virus at 25°C. Table 1 shows the growth properties of four wild-type viruses, i.e. A/Texas/1/77, A/Victoria/3/75, A/USSR/92/77, A/Ann Arbor/6/60, the cold-adapted A/Ann Arbor/6/60, and the recombinant DU 5 clone 15 at 25°C, 33°C, 37°C, 38°C and 39°C. The results shown were obtained by infecting MDCK cells. The growth properties in chick embryo kidney cells (not shown) were similar to those in MDCK cells. The actual selection for avirulence at 25°C is unknown, as most wild-type viruses do grow well in our tissue culture system at this temperature. This has also been recognized by Van Kirk et al. [28], but is in contrast with the findings of Spring et al. [26]. Nevertheless, by performing the recombination procedure at 25°C, there seems to be a selection of certain recombinants. Cox et al. [5] found only four out of 64 theoretically possible different recombinants by performing the recombination at 25°C with A/Ann Arbor/6/60 antiserum, which is in accordance with our own findings.

DU 5 clone 15 was selected for the production of vaccine. This clone has the following characteristics. 1. The haemagglutinin and neuraminidase are derived from the wild-type A/USSR/92/77 ($H_1N_1$) strain, as demonstrated in HI and NI tests. 2. The genes

TABLE 1

Growth properties at 25°C, 33°C, 37°C, 38°C and 39°C and shutoff temperature of wild-type and cold-adapted viruses

| | | $^{10}$Log of virus growth (p.f.u./ml) at | | | | | Shutoff temp (°C) |
|---|---|---|---|---|---|---|---|
| | | 25°C | 33°C | 37°C | 38°C | 39°C | |
| A/Ann Arbor/6/60 | WT | 7.30 | 7.95 | 8.20 | 8.11 | 6.43 | > 39 |
| A/USSR/92/77 | WT | 6.00 | 8.00 | 7.90 | 7.41 | 6.78 | > 39 |
| A/Victoria/3/75 | WT | 6.41 | 7.23 | ND | 6.34 | 6.30 | > 39 |
| A/Texas/1/77 | WT | 6.90 | 7.89 | 8.00 | 7.69 | 7.57 | > 39 |
| A/Ann Arbor/6/60 | CA | 7.67 | 8.38 | 7.49 | 6.23 | < 3.00 | 38 |
| DU 5 clone 15 | CA | 6.00 | 8.15 | 7.60 | 5.30 | < 3.00 | 38 |

The shutoff temperature is defined as the temperature at which a 100-fold reduction in viral titre can be observed as compared to the titre at 33°C (ND = not done) WT = wilde-type, CA = cold-adapted

coding for $P_1$, $P_2$, $P_3$, NP, M and NS proteins are derived from the cold-adapted A/Ann Arbor/6/60 strain. From Fig. 1 we can conclude that the $P_1$, $P_2$, M and NS proteins of the recombinant originate from the cold-adapted A/Ann Arbor/6/60 strain. From RNA gels (not shown) we conclude that the $P_3$ and NP genes of the recombinant originate from the cold-adapted A/Ann Arbor/6/60 strain. 3. The replication of DU 5 clone 15 in the lungs of hamsters is restricted to the same degree as the cold-adapted A/Ann Arbor/6/60 virus, while the wild-type virus A/USSR/92/77 grew in the lungs of hamsters to high titres (Fig. 2). 4. The cold-adapted A/Ann Arbor/6/60 donor strain is a temperature-sensitive (*ts*) strain; its growth is considerably reduced at temperatures above 38°C (shutoff temperature). The A/USSR/92/77 strain does not show a specific shutoff temperature. The shutoff temperature of DU 5 clone 15 is the same as that of the cold-adapted A/Ann Arbor/6/60 (Table 1).

*Clinical signs and symptoms after vaccination*

All clinical reactions after administration of the above mentioned vaccine or placebo were mild (Fig. 3). No pronounced increase in clinical signs or symptoms was observed after vaccination in comparison with the pre-vaccination level (day 0). The most frequently observed clinical signs and symptoms were 'stuffy nose' and 'rhinorrhoea'. Stuffy nose occurred for one or more days in five volunteers in the vaccine group and in four volunteers in the placebo group. Rhinorrhoea occurred for one or more days in five volunteers in the vaccine group and in three volunteers in the placebo group. It should be noted, however, that prior to vaccination, stuffy nose was present in two volunteers in the vaccine group and in one volunteer in the placebo group; rhinorrhoea was observed prior to vaccination in three volunteers of the vaccine group and in two volunteers of the placebo group. Thus, these clinical signs and symptoms occurred in similar numbers in both groups.

The body temperature of the volunteers was determined prior to vaccination and for 5 days after vaccination. No clinical difference could be observed on any day between the mean temperature in the vaccine group and that of the placebo group, the greatest difference being 0.2°C. No body temperature above 37.8°C was observed in any individual after vaccination.

We conclude that in this limited study there were no indications of vaccine-related unwanted effects.

*Serum antibody response*

Table 2 presents the serum HI antibody response against A/USSR/92/77 ($H_1N_1$). Both subjects with and subjects without prevaccination antibodies showed a response. The geometric mean antibody level in the vaccine group showed, after one administration of vaccine, a mean 2.1-fold increase and after two administrations, a mean 3.9-fold increase. Rises $\geqslant$ 4-fold after one administration were observed in three out of

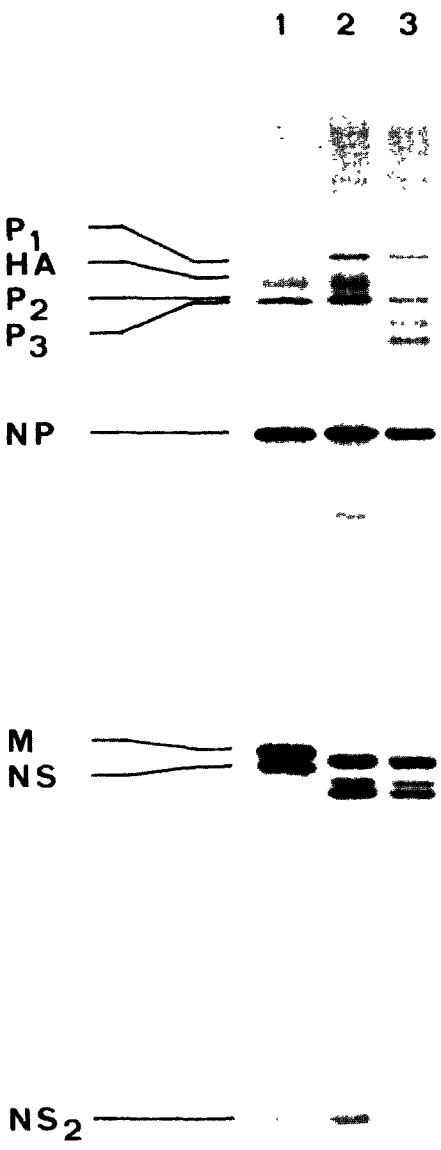


Fig 1. Polyacrylamide gel electrophoresis of [$^{35}$S]methionine-labelled proteins of parent strain A/USSR/92/77 (lane 1), recombinant DU 5 clone 15 (lane 2), and parent strain A/Ann Arbor/6/60 CA (lane 3). From this autoradiogram we conclude that the $P_1$, $P_2$, M and NS proteins of the recombinant originate from the cold-adapted A/Ann Arbor/6/60 strain

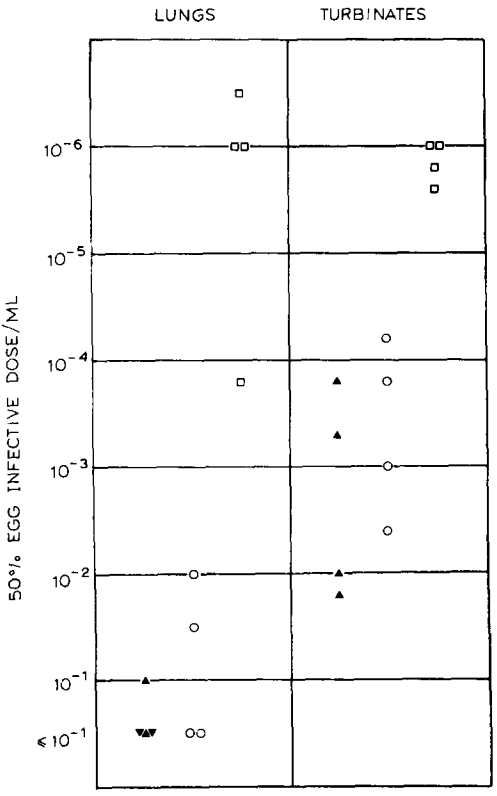


Fig. 2. Growth properties of DU 5 clone 15 (▲), and parent viruses A/Ann Arbor/6/60 CA (○) and A/USSR/92/77 (□) in lungs and turbinates of hamsters. Lung and nasal tissue are removed 48 h after infection. The viral content of the tissues was determined by titrations in eggs

14 vaccines, while after two vaccinations, a total of eight out of 14 showed 4-fold rises. The placebo response was to all intents and purposes nil after both administrations.

In the vaccine group, the mean serum NI-antibody level (see Table 2) against A/USSR/92/77 ($H_1N_1$) after one administration of vaccine showed a mean 1.3-fold increase (from 2.5 to 3.2) and after two administrations a mean 2.0-fold increase (from 2.5 to 5.0). NI-antibody titrations, using a strain with a different haemagglutinin ($H_{eq1}N_1$), gave similar results (not shown). Again, placebo response was virtually nil after both administrations.

These results can only be seen as an indication of the immunogenic properties of the vaccine. Studies with larger populations are necessary to measure reliably the immunogenicity.

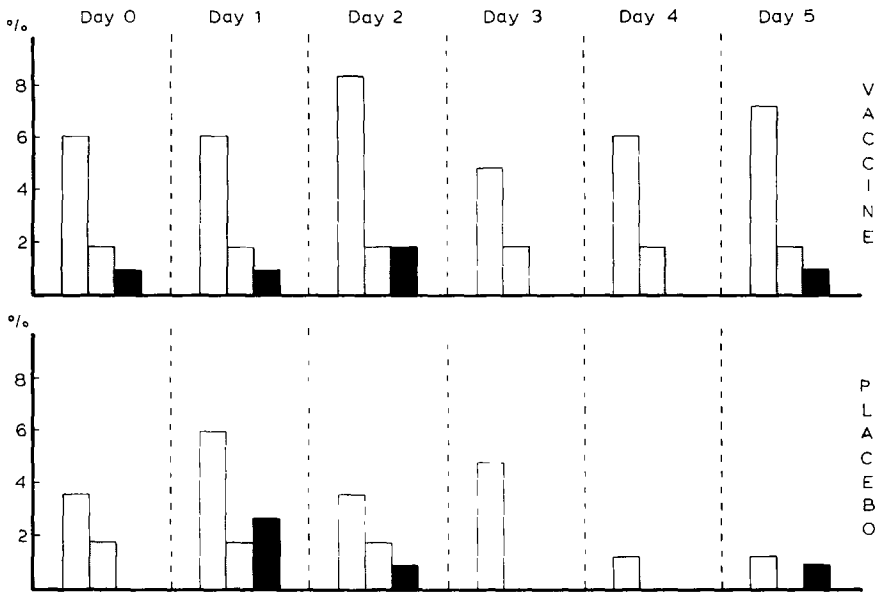


Fig 3. The incidence of unwanted clinical signs and symptoms immediately before vaccination (day 0) and for 5 days after vaccination, expressed as a percentage of the theoretically possible maximum. Upper respiratory tract symptoms (□) looked for are stuffy nose, rhinorrhoea, conjunctivitis, laryngitis, throat injection, swollen uvula Mid-respiratory tract symptoms ( ) looked for are cough, pain during coughing, dyspnea, rhonchi Systemic illnesses (■) looked for are chills, headache, myalgia, joint pains, malaise, nausea, vomiting, diarrhoea The percentage on the ordinate, e g for upper respiratory piratory tract symptoms on day 0 (pre-vacc.) in the vaccine group is calculated as follows

$$\frac{\text{sum of all upper resp. tract symptoms observed}}{\text{(No of upper resp tract symptoms looked for)} \times \text{(No. of volunteers)}} \times 100 =$$

$$\frac{2 \text{ stuffy nose} + 3 \text{ rhinorrhoea} = 5}{6 \times 14} \times 100 = 6\%$$

All signs and symptoms in all subjects would result in 100%

*Transmission of vaccine virus in human volunteers*

Virus was isolated in nine out of 14 vaccinees (Table 3) and in none of the placebo group. Moreover, the first vaccination induced a geometric mean rise in serum HI-antibody titres from 19 to 40 in the vaccine group (Table 2), while no increase was observed in any of the 14 placebo-treated volunteers. From these results, it could be concluded that there were no signs of transmission of the vaccine virus to non-vaccinated persons.

TABLE 2

Serum HI- and NI-antibody titres against A/USSR/92/77 before vaccination, after one vaccination and after two vaccinations

| | Subject No | I Pre-vacc. | II Post 1st vacc Pre 2nd vacc | III Post 1st vacc | Mean-fold increase I → II | Mean-fold increase I → III | Virus isolated from nasal swabs |
|---|---|---|---|---|---|---|---|
| Serum HI antibody titres | | | | | | | |
| Vaccine group | 3642 | 8 | 8 | 30 | 1 0 | 3.8 | + |
| | 3646 | 8 | 8 | 32 | 1.0 | 4 0 | - |
| | 3647 | 68 | 136 | 271 | 2 0 | 4 0 | + |
| | 3651 | 8 | 128 | 242 | 16.0 | 30.3 | + |
| | 3652 | 8 | 8 | 8 | 1 0 | 1.0 | + |
| | 3653 | 128 | 162 | 256 | 1 3 | 2 0 | - |
| | 3656 | 8 | 16 | 32 | 2.0 | 4.0 | + |
| | 3657 | 8 | 91 | 81 | 11 4 | 10.1 | + |
| | 3659 | 8 | 8 | 32 | 1 0 | 4 0 | + |
| | 3661 | 14 | 8 | 14 | 0.6 | 1 0 | + |
| | 3662 | 8 | 64 | 181 | 8 0 | 22.6 | - |
| | 3664 | 128 | 484 | 543 | 3.8 | 4 2 | - |
| | 3666 | 32 | 68 | 91 | 2.1 | 2.8 | + |
| | 3669 | 68 | 81 | 91 | 1.2 | 1 3 | - |
| | Geom. mean | 19 | 40 | 73 | 2 1 | 3.9 | |
| Placebo group | Geom. mean | 19 | 19 | 21 | 1.0 | 1.1 | |
| Serum NI antibody titres | | | | | | | |
| Vaccine group | 3642 | 1 | 1 | 4 | 1 | 4 | |
| | 3646 | 1 | 1 | 1 | 1 | 1 | |
| | 3647 | 7 | 8 | 15 | 1 1 | 2.1 | |
| | 3651 | 1 | 1 | 5 | 1 | 5 | |
| | 3652 | 1 | 1 | 1 | 1 | 1 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 3653 | 11 | 15 | 18 | 1 4 | 1.6 |
| | 3656 | 1 | 1 | 1 | 1 | 1 |
| | 3657 | 1 | 3 | 4 | 3 | 4 |
| | 3659 | 1 | 1 | 5 | 1 | 5 |
| | 3661 | 10 | 12 | 9 | 1 2 | 0 9 |
| | 3662 | 2 | 4 | 8 | 2 | 4 |
| | 3664 | 7 | 21 | 23 | 3 | 3 3 |
| | 3666 | 5 | 6 | 5 | 1.2 | 1 |
| | 3669 | 6 | 6 | 7 | 1 | 1.2 |
| | Geom. mean | 2.5 | 3.2 | 5.0 | 1 3 | 2 0 |
| Placebo group | Geom mean | 2.1 | 2.0 | 2 0 | 0 9 | 1 0 |

With the titration method used [27], an antibody titre of 100–150 is regarded as a protective level [29] In the last column are indicated those subjects from which virus could be reisolated from nasal swabs

TABLE 3

Virus isolations from nasal swabs taken after the first vaccination

| Subject no | Amount of virus in nasal swabs (expressed as log $EID_{50}$/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day after vaccination | | | | | | |
| | 0 | 1 | 2 | 3 | 5 | 7 | 9 |
| 3642 | - | 1 36 | 1 60 | - | - | - | - |
| 3647 | - | - | ⩽1 00 | - | - | - | - |
| 3651 | - | 3 80 | 4 00 | 2 50 | - | - | - |
| 3652 | - | 1 16 | 1 40 | 2 60 | 2 38 | * | - |
| 3656 | - | - | 1 75 | ⩽1 00 | - | - | - |
| 3657 | - | * | ⩽1 00 | 1 25 | - | - | - |
| 3659 | - | 1 60 | 2 25 | 1.75 | 1 83 | - | - |
| 3661 | - | - | - | 3 25 | ⩽1.00 | - | - |
| 3666 | - | * | - | - | - | - | - |

Nasal swabs who were negative ( $\leqslant 10^1$ $EID_{50}$/ml) were passaged a second time on eggs The isolates who were positive after a second passage are indicated (*). The nasal swabs in which no virus could be shown after two passages are presented as negative (-). The nasal swabs from other participants were all negative after two passages

*Genetic stability of the vaccine virus during in vivo replication*

The genetic stability of the vaccine virus was assessed by characterization of the isolated virus from the nasal swabs with regard to haemagglutinin and neuraminidase identity, temperature sensitivity in tissue culture, and growth properties in upper and lower respiratory tracts of hamsters. The viral titres in the nasal swabs (maximal at day 2 or day 3 after vaccination, Table 3) indicate that the isolated viruses are not likely to be residual vaccine viruses.

The haemagglutinin and neuraminidase proteins of the isolated viruses were identified in the HI and NI test as $H_1$ and $N_1$, in all nine cases.

Virus isolates from all nine volunteers showed a shutoff temperature in MDCK tissue culture cells of 38°C. Thus, the temperature-sensitive properties of all isolates closely resemble that of the A/Ann Arbor/6/60 donor strain and that of the vaccine virus.

The viruses isolated from the nine volunteers were further characterized in the hamster model. The growth properties were as follows. The virus isolates from seven volunteers did not grow in hamster lungs ($\leqslant 10^{1.0}$ $EID_{50}$/ml) and the virus isolates from the remaining two volunteers grew only to a concentration varying from $10^{1.0}$ to $10^{1\,33}$ $EID_{50}$/ml in hamster lungs. This is comparable to the original vaccine virus (Fig. 2) and far below the concentration of about $10^6$ $EID_{50}$/ml, which characterizes wild-type influenza viruses. Virus growth was observed in the hamster turbinates, where titres varied from $10^{1\,2}$ to $10^{3\,5}$ $EID_{50}$/ml (mean of 4 determinations per subject), indicating that the isolated viruses grew well in the turbinates, as would be expected.

## DISCUSSION

The factors responsible for the attenuation of the cold-adapted A/Ann Arbor/6/60 virus are not yet completely understood, but probably multiple lesions in the genome are involved [12]. In view of this, an extensive characterization of each recombinant using the A/Ann Arbor/6/60 as a donor strain is still required including, if possible, a complete genotyping. One clinical study with a similar dose level of a recombinant of A/Ann Arbor/6/60 with the A/Scotland/840/74 ($H_3N_2$) strain (CR18 clone 7) showed an improper attenuation, causing a febrile reaction (39°C) in one of the volunteers [17]. Characterization of the cold recombinant used in that study, showed that it still contained the NS gene from the A/Scotland/840/74 wild type [5].

The cold-recombinant reported in the present study was a cold-adapted temperature-sensitive strain with the genes coding for the $P_1$, $P_2$, $P_3$, NP, M and NS proteins from the cold-adapted A/Ann Arbor/6/60 donor strain and the genes coding for the HA and NA proteins from the A/USSR/92/77 strain. This is theoretically the optimal combination, provided that the genes for the surface proteins do not contribute to the attenuated properties. After passing appropriate selection criteria, the recombinant was tested clinically in volunteers (most of whom where unprimed), to assess the degree of attenuation and to obtain evidence on the reliability of this method of producing seed virus for live influenza vaccine.

One of the requirements for an acceptable live influenza vaccine is that it does not spread from vaccinees to other persons. The absence of an antibody response in the placebo group and the absence of virus in nasal swabs in these subjects show clearly that there was no detectable transmission of virus. As the first 10 days of the study were performed under conditions of close household contact between vaccine group and placebo group, a possible transmission was likely to be detected. No virus shedding was observed after day 7, which also minimizes the risk of transmission.

The incidence and severity of local and systemic unwanted effects after administration of vaccine was very low and clinically insignificant. There was no evidence of vaccine-related unwanted effects. No febrile reactions were observed after administration of vaccine. This indicated that this recombinant of A/Ann Arbor/6/60 and A/USSR/92/77 is sufficiently attenuated, at least in this study population of 14 healthy volunteers. On the other hand, the wild-type parent strain A/USSR/92/77 is not very virulent [9, 24], which makes it difficult to conclude in a clinical study whether the avirulence is derived from the A/Ann Arbor/6/60 genes.

The vaccine virus proved to be genetically stable. The virus isolated from nasal swabs of vaccinees was in all nine cases identical to the original vaccine virus with regard to haemagglutinin and neuraminidase identity, temperature sensitivity and growth properties in upper and lower respiratory tracts of hamsters.

The serum antibody response is difficult to evaluate in view of the small number of participants. Both vaccinations induced a response in the vaccine group. The mean serum HI antibody titre in the vaccine group rose overall from 19 to 73 after two vaccinations

(Table 2), which is a moderate antibody response in comparison with the antibody responses usually obtained with inactivated influenza vaccines. This might be due to the fact that most of the volunteers were unprimed for this strain. However, a lower serum antibody response with live influenza vaccine has previously been observed [2, 8]. It remains uncertain whether the serum HI-antibody level properly reflects the state of immunity after vaccination with live vaccine. Cell-mediated immunity and local antibodies will also be of importance. For instance, volunteers Nos. 3652 and 3661 did not give any serum antibody response in the HI or the NI test (Table 2). However, the vaccine virus had grown well in these volunteers as detected by the nasal swabs (Table 3). Since this in vivo replication is comparable with a normal infection, it is likely that these two volunteers are immune to subsequent infection.

The results in this pilot study indicate that this candidate vaccine can be used safely in larger studies, to investigate the proper dose with regard to efficacy and reactogenicity. Although the future of live influenza vaccines might look promising, there still exist some problems, such as the possibility of interference between two influenza A strains or between an A and a B strain in a polyvalent vaccine [25]. Another problem is a shift situation where after vaccination a co-infecting virulent influenza particle can pick up the genes for the surface proteins of a vaccine virus, thus creating the very epidemic it is desired to prevent.

We feel, however, that the main problem in manufacturing live influenza vaccines, in comparison with inactivated vaccines, is the long period of time necessary to produce, control and test (clinically) a new influenza variant in a live vaccine before it can be licensed [7,18]. Although this problem is recognized in the recommended regulations, it makes the usually rapid updating of the vaccine impossible. This implies that in the case of a new strain, live influenza virus vaccine runs one season behind compared to the inactivated vaccines. In our view this is unacceptable, and we are therefore in serious doubt whether under these conditions it will be possible for live influenza vaccine to replace the current inactivated vaccines for yearly vaccination purposes.

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REFERENCES

1 Aymard-Henry, M , Coleman, M T , Dowdle, W.R., Laver, W G., Schild, G C and Webster, R G. (1973) Influenza virus neuraminidase and neuraminidase-inhibition test procedures Bull W H.O 48, 199–202

2 Beare, A.S , Hobson, D , Reed, S E and Tyrrell, D A J (1968) A comparison of live and killed influenza virus vaccines. Lancet 2, 418–420.

3 Bonner, W.M. and Laskey, R A (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem 46, 83–88.

4 Jawitz, E , Melnick, J L. and Adelberg, E A. (eds.) (1976) Calculation of neutralized virus $LD_{50}$ by the Reed-Muench method In Review of Medical Microbiology, 12th ed. (Lange Medical Publications, Los Altos, CA ) p. 338

5 Cox, N J., Maassab, H F. and Kendal, A P (1979) Comparative studies of wild-type and cold-mutant (temperature-sensitive) influenza viruses nonrandom reassortment of genes during preparation of live virus vaccine candidates by recombination at 25° between recent $H_3N_2$ and $H_1N_1$ epidemic strains and cold-adapted A/Ann Arbor/6/60. Virology 97, 190–194.

6 Davenport, F M., Hennessy, A.V , Maassab, H.F , Minuse, E., Clark, L.C , Abrams, G D and Mitchell, J.R. (1977) Pilot studies on recombinant cold-adapted live type A and B influenza virus vaccines. J Infect Dis 136, 17–25

7 Ennis, F., Higy-Mandic, L., Huygelen, C , Perkins, F.T , Van Ramshorst, J.D., Schild, G , Smith, C. and Weisz-Malecek, R. (1979) Requirements for influenza vaccine (live) W.H O Requirements for Biological Substances, No. 28, pp. 148–191.

8 Freestone, D S., Hamilton-Smith, S , Schild, G.C., Buckland, R , Chinn, S. and Tyrrell, D A J. (1972) Antibody responses and resistance to challenge in volunteers vaccinated with live attenuated, detergent split and oil adjuvant $A_2$/Hong Kong/68 ($H_3N_2$) influenza vaccines J. Hyg (Camb.) 70, 531–543

9 Glass, R I.M., Brann, E.A , Slade, J D., Jones, W.E , Scally, M J., Craven, R B and Gregg, M.B (1978) Community-wide surveillance of influenza after outbreaks due to $H_3N_2$ (A/Victoria/75 and A/Texas/77) and $H_1N_1$ (A/USSR/77) influenza viruses, Mercer County, New Jersey 1978 J. Infect Dis. 138, 703–706

10 Hrabar, A , Vodopija, I , André, F.F., Mitchell, J.R., Maassab, H.F , Hennessy, A.V and Davenport, F.M (1977) A placebo-controlled dose-response study of the reactogenicity and immunogenicity of a cold-adapted recombinant A/Victoria/3/75 ($H_3N_2$) live influenza virus candidate vaccine in healthy volunteers Dev Biol. Standard 39, 53–60

11 Inglis, S.C , Carroll, A.R , Lamb, R S. and Mahy, B.W.J (1976) Polypeptides specified by the influenza virus genome Virology 74, 489–503

12 Kendal, A.P., Cox, N J , Galphin, J C and Maassab, H.F. (1979) Comparative studies of wild-type and cold-mutant (temperature-sensitive) influenza viruses independent segregation of temperature-sensitivity of virus replication from temperature-sensitivity of virion transcriptase activity during recombination of mutant A/Ann Arbor/6/60 with wild-type $H_3N_2$ strains. J Gen Virol. 44, 443–456.

13 Kitayama, T , Togo, Y , Hornick, R.B and Friedwald, W T. (1973) Low-temperature-adapted influenza A2/AA/6/60 virus vaccine in man. Infect. Immun. 7, 119–122

14 Maassab, H F (1967) Adaptation and growth characteristics of influenza virus at 25°C, Nature 213, 612–614

15 Maassab, H.F (1973) Properties of influenza virus 'cold' recombinants In Negative Strand Viruses, Vol 2 Eds. B.W.J. Mahy and R D. Barry (Academic Press, London) pp 755–763.

16 Mackenzie, J S. (1977) The potential advantages and requirements of live attenuated influenza virus vaccines Aust N Z.J Med 7, 431–437

17 Murphy, B R , Holley Jr., H P., Berquist, E J , Levine, M M., Spring, S B., Maassab, H F., Kendal, A.P and Chanock, R M (1979) Cold-adapted variants of influenza A virus evaluation in adult seronegative volunteers of A/Scotland/840/74 and A/Victoria/3/75 cold-adapted recombinants derived from the cold-adapted A/Ann Arbor/6/60 strain Infect Immun 23, 253–259

18 National Institute for Biological Standards and Control Suggested regulations for the manufacture and testing of live attenuated influenza vaccines Holly Hill, London

19 Oxford J S., McGeoch, D.J., Schild, G C. and Beare, A S. (1978) Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. Nature 273, 778–779.
20 Palese, P and Schulman, J.L (1976) Differences in RNA patterns of influenza A viruses. J Virol 17, 876–884.
21 Scholtissek, C. (1978) The genome of the influenza virus Curr. Top Microbiol. Immunol 80, 139–169
22 Scholtissek, C., Harms, E., Rohde, W , Orlich, M and Rott, R (1976) Correlation between RNA fragments of fowl plague virus and their corresponding gene functions Virology 74, 332–344
23 Scholtissek, C., Vallbracht, A , Flehmig, B and Rott, R (1979) Correlation of pathogenicity and gene constellation of influenza A virus. Virology 95, 492–500
24 Shortridge, K F and Osmund, I (1978) $H_1 N_1$ influenza in Hong Kong Lancet 1, 322
25 Spencer, M.J , Cherry, J.D., Powell, K.R. and Sumaya, C.V (1979) A clinical trial with Alice/R-75 strain, live attenuated serum inhibitor-resistant intranasal bivalent influenza A/B vaccine Med Microbiol Immunol 167, 1–9.
26 Spring, S.B , Maassab, H F., Kendal, A P , Murphy, B.R. and Chanock, R M (1977) Cold adapted variants of influenza A Arch. Virol. 55, 233–246.
27 Van der Veen, J. and Mulder, J. (1950) Studies on the Antigenic Composition of Human Influenza Virus A Strains with the Aid of the Haemagglutination Inhibiting Technique (H.E. Stenfert Kroese, Leiden).
28 Van Kirk, J.E , Mills, J. and Chanock, R.M (1971) Evaluation of low temperatures grown influenza $A_2$/Hong Kong virus in volunteers Proc Soc Exp. Biol. Med 136, 34–41.
29 Wesselius-De Casparis, A , Masurel, N and Kerrebijn, K.F (1972) Field trial with human and equine influenza vaccines in children protection antibody titres. Bull. W.H O 46, 151–157.