

# Immune Response to Vaccination with a Live Influenza Virus (H<sub>3</sub>N<sub>2</sub>) Vaccine ("Ann" Strain)

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A live attenuated influenza virus ("Ann" strain) derived from A/England/878/69 was given intranasally to a group of volunteers, most of whom had already circulating antibodies against H<sub>3</sub>N<sub>2</sub> viruses at the time of inoculation. There was a fourfold or higher increase of circulating hemagglutination-inhibiting antibodies in those volunteers who had relatively low initial titers. The response was lower in those with initially higher serum titers. The pattern of the serum neutralizing antibody response was very similar. The geometric means of the antineuraminidase antibodies were 67 and 118 pre- and postvaccination, respectively. All subjects showed a rise in local neutralizing antibodies in their nasal secretions with geometric means of 4 and 17 pre- and postvaccination, respectively. The levels of local antineuraminidase antibodies also rose in most subjects. In addition to the response to the homologous virus type, the antibody formation to the recent A/England/42/72 was measured in the sera and nasal secretions of some subjects. There was a clearcut response in most of the sera and in all of the secretions examined. The stimulation of circulating lymphocytes was measured in 6 volunteers. All volunteers showed a temporary stimulation. The stimulation index ranged between 2.5 and 28.5.

Several different methods have been used for the attenuation of influenza A virus strains, and various marker systems of attenuation have been studied (3, 5, 15, 16). Asian (H<sub>2</sub>N<sub>2</sub>) virus strains which were resistant to serum inhibitors were shown to be attenuated for man (3). This principle has also been applied to the Hong Kong strain (H<sub>3</sub>N<sub>2</sub>). Beare and Bynoe (4) obtained a H<sub>3</sub>N<sub>2</sub> strain which was partially resistant to serum inhibitors and which showed decreased virulence for man, but its attenuation was incomplete. In our laboratory, a Hong Kong strain was made completely resistant to inhibitors and it was shown to have an acceptable degree of attenuation for man (12, 14, 17). In the present study the nasal and circulating antibody response to intranasal administration of this strain ("Ann" strain) was investigated in a group of volunteers. Antibodies were measured against early Hong Kong virus strains but also against the recent A/England/42/72 variant, in order to evaluate the degree of immunological cross-reaction between the Ann strain and the new variant.

## MATERIALS AND METHODS

**Vaccine.** A freeze-dried batch (RLB 101/21/2) of the Ann strain NRRL 5640 was used in this experi-

ment. A dose of 10<sup>7.4</sup> mean egg infective doses (EID<sub>50</sub>) was given intranasally as drops in a total volume of 0.5 ml (0.25 ml to each nostril). A second dose was given 16 days later. The vaccine was reconstituted with distilled water just before use. This batch had been tested for safety and potency as described elsewhere (12).

**Study group.** Ten male and two female volunteers between 20 and 33 years old were used in this study. Blood samples were obtained just before inoculation and 16 and 30 days after inoculation. The participants were asked to note any symptoms occurring during the 30-day observation period and to take their temperature daily for the first 4 days after each inoculation.

**Nasal washings.** Nasal washings were obtained by the method described by Rossen et al. (20). A volume of 6 to 10 ml was instilled into each nostril while the subject was in the sitting position with the head in hyperextension. The subjects then forcefully expelled the fluid with varying amounts of mucus. This procedure was repeated twice using the same fluid, and the combined effluent volume varied between 10 and 15 ml. All samples were homogenized by sonic treatment and then freeze-dried.

Total protein, immunoglobulin (Ig) A and G concentrations were determined in all samples before and after lyophilization. Lyophilization resulted in a loss of about 70% of the initial amount of IgA. After reconstitution, all samples were adjusted to a concentration of 200 µg of secretory IgA per ml. The samples

were tested for the presence of blood, using the Hemastix method (Hemastix, Ames, Iowa).

**Antibody assays.** All tests were done on paired samples. Two recombinant viruses were used in the tests: X-31 which has the antigenic composition of the 1968 Hong-Kong strain (13) and MRC-2 which has the antigenic composition of the A/England/42/72 (G. C. Schild, personal communication).

For the hemagglutination-inhibition tests (HI), all sera were inactivated at 56 C for 30 min and treated with a 25% suspension of Kaolin (21). Four hemagglutinating units were mixed with twofold serum dilutions in tubes and left in contact for 1 h at room temperature. A 0.5% suspension of chicken erythrocytes was then added. All titers were expressed as reciprocals of the dilution at which the hemagglutination was completely inhibited.

For the neutralization tests, we used a modification of the membrane-on-shell method described by Fazekas de St. Groth and White (8). Thirty to 100 mean infective doses (ID<sub>50</sub>) of virus were mixed with equal volumes of dilutions of serum or nasal samples and left in contact for 1 h at 37 C. A 0.1-ml amount of the mixture was then inoculated into tubes containing pieces of egg shells with the chorioallantoic membrane attached to it. After 2 days the multiplication of the influenza virus was checked by adding one drop of a 2% suspension of chicken red blood cells directly to the tubes after removal of the shells. All titers were expressed as reciprocals of the dilution at which a complete neutralization was observed.

The method recommended by the World Health Organization Influenza Centre was used for the determination of the neuraminidase-inhibiting antibodies (Influenza Reagents, neuraminidase inhibition test, WHO World Influenza Centre, London). Allantoic fluid injected with the X-31 strain was used in all tests. The mixtures of virus and serum or nasal samples were left at room temperature for 3 h. They were then incubated for 18 h at 37 C and examined for free *N*-acetylneuraminic acid by the method of Warren, as modified by Aminoff (1).

**Assay of lymphocyte stimulation.** Heparinized blood samples were obtained by venipuncture on days 0, 15, and 30 after the first inoculation. Lymphocytes were separated from red blood cells by sedimentation of the latter by high-molecular-weight dextran and washed in phosphate-buffered saline. The lymphocytes were resuspended at a final concentration of 10<sup>6</sup> cells/ml in Eagle basal medium containing 20% inactivated fetal calf serum, and the viral antigen was added in dilutions varying between 10<sup>-2</sup> and 10<sup>-6</sup>; 0.1 ml was added to each culture. The viral antigen consisted of an ultraviolet-inactivated suspension of Ann strain with an initial titer of 10<sup>7.7</sup> EID<sub>50</sub>/ml (19). Phytohemagglutinin was used as a nonspecific positive control and physiological saline as a negative control. After incubation in a humidified CO<sub>2</sub> incubator for 144 h (24 to 48 h for the phytohemagglutinin-stimulated cultures), the stimulation of deoxyribonucleic acid synthesis was tested by measuring the incorporation of tritiated thymidine into trichloroacetic acid-precipitable material. One  $\mu$ Ci (Radiochemical Centre, Amersham, England) was added per culture and left in contact for the last 24 h of the

incubation period. Tests were run in duplicate or in triplicate. The results are expressed as stimulation indices, i.e., the ratio of mean counts per minute in the material under test compared to the mean counts per minute in the controls. The virus dilution which produced the highest stimulation varied between 10<sup>-2</sup> and 10<sup>-6</sup> according to the subjects examined.

## RESULTS

**Clinical reactions.** No significant symptoms were observed in any of the vaccinees. Subject no. 13 reported some sneezing on day 2 after the first dose and subject no. 4 had a "stuffy" nose for 1 day after the second dose.

**Antibody response.** All results of antibody determinations are summarized in Table 1. The circulating HI antibodies against X-31 pre- and post-vaccination were determined in all 12 subjects. All but one (no. 6) were seropositive at the time of vaccination; the titers of the seropositives ranged between 16 and 128. All 4 subjects with a titer of  $\leq 32$  to the X-31 virus showed a fourfold or greater antibody increase. Six subjects had a titer of 64 prevaccination. Four of them showed an increase of only one dilution after vaccination. The 2 others showed a fourfold rise. The 2 subjects with an initial titer of 128 showed a twofold increase. The geometric means of the HI titers were 47 and 120 pre- and postvaccination, respectively.

The sera of all 12 volunteers were also tested for antibodies against the A/England/42/72 variant. All prevaccination titers ranged between < 8 and 32, and all postvaccination titers between 16 and 128. Ten out of 12 subjects showed a fourfold or greater increase, whereas the 2 remaining subjects showed a twofold difference. The geometric means were 11 and 50 before and after vaccination, respectively.

The neutralization (N) titers to the X-31 virus were determined in the sera of 6 subjects. As shown in Table 1, they correlated well with the HI titers. The sera of 6 volunteers were tested for N antibodies against MRC-2; 5 of them showed a fourfold or greater titer increase. The remaining 1 volunteer showed an increase of one dilution only.

The circulating anti-neuraminidase (AN) antibodies to X-31 were determined in all subjects before and after vaccination. All titers were relatively high initially. The geometric means were 67 and 118, respectively, in the pre- and postvaccination sera.

In the local antibody studies the N antibody response was determined against both X-31 and MRC-2. All 12 subjects showed a rise in N antibodies against X-31; the geometric means were 4 and 17 before and after vaccination, respectively.

TABLE 1. Nasal and circulating antibody response in volunteers vaccinated intranasally with the "Ann" strain

| Subject no.       | Circulating antibodies to: |                |                 |                    |                 | Nasal antibodies to: |    |                    |
|-------------------|----------------------------|----------------|-----------------|--------------------|-----------------|----------------------|----|--------------------|
|                   | X-31 <sup>a</sup>          |                |                 | MRC-2 <sup>a</sup> |                 | X-31 <sup>a</sup>    |    | MRC-2 <sup>a</sup> |
|                   | HI <sup>b</sup>            | N <sup>b</sup> | AN <sup>b</sup> | HI                 | N               | N                    | AN | N                  |
| 1 Pre             | 32                         | 32             | 40              | 16                 | 16              | <4                   | <2 | ≤4                 |
| Post <sup>c</sup> | 128                        | 128            | 80              | 64                 | 256             | 6                    | 4  | 6                  |
| 4 Pre             | 64                         | 32             | 80              | 16                 | NT <sup>d</sup> | <4                   | ≤4 | NT                 |
| Post              | 256                        | 256            | 160             | 128                | NT              | 32                   | 12 | NT                 |
| 6 Pre             | <16                        | 16             | 80              | <16                | <8              | 4                    | <2 | <2                 |
| Post              | 256                        | 256            | 320             | 128                | 64              | 32                   | 8  | 32                 |
| 9 Pre             | 64                         | NT             | 160             | 32                 | NT              | 16                   | 4  | NT                 |
| Post              | 256                        | NT             | 320             | 128                | NT              | 24                   | 4  | 48                 |
| 11 Pre            | 128                        | NT             | 160             | 16                 | NT              | 6                    | 6  | NT                 |
| Post              | 256                        | NT             | 320             | 128                | NT              | 12                   | 6  | NT                 |
| 12 Pre            | 16                         | 32             | 80              | <16                | 8               | 12                   | ≤2 | NT                 |
| Post              | 256                        | ≥256           | 320             | 32                 | 128             | 20                   | 4  | NT                 |
| 13 Pre            | 128                        | NT             | 320             | 16                 | NT              | 6                    | 4  | NT                 |
| Post              | 256                        | NT             | ≥640            | 64                 | NT              | 16                   | 6  | 12                 |
| 14 Pre            | 64                         | NT             | 160             | 8                  | NT              | ≤4                   | 12 | NT                 |
| Post              | 128                        | NT             | 320             | 16                 | NT              | 10                   | 12 | NT                 |
| 15 Pre            | 64                         | 64             | 80              | <8                 | 32              | <4                   | NT | NT                 |
| Post              | 128                        | 128            | 80              | 16                 | 64              | 12                   | 6  | 24                 |
| 17 Pre            | 32                         | 32             | 40              | 8                  | 16              | <4                   | <4 | NT                 |
| Post              | 256                        | ≥256           | 160             | 64                 | 256             | 32                   | 4  | 64                 |
| 18 Pre            | 64                         | NT             | 160             | 8                  | NT              | ≤4                   | <4 | 8                  |
| Post              | 128                        | NT             | 160             | 32                 | NT              | 24                   | 6  | 48                 |
| 22 Pre            | 64                         | NT             | 40              | 8                  | <8              | 12                   | NT | ≤2                 |
| Post              | 128                        | NT             | 40              | 16                 | 16              | 24                   | NT | 8                  |

<sup>a</sup> X-31, Recombinant with antigenic composition of A/Hong-Kong/1/68. MRC-2, Recombinant with antigenic composition of A/England/42/72.

<sup>b</sup> HI, Hemagglutination-inhibition tests; N, neutralization tests; AN, antineuraminidase tests.

<sup>c</sup> 30 days after 1st dose of vaccine.

<sup>d</sup> NT, Not tested.

In 4 subjects the N antibody titer to MRC-2 was determined in both pre- and postinoculation sera and all 4 showed an increase. In 4 other subjects, only the postvaccination sera could be tested, because not enough material was left over in the prevaccination samples. The titers in the postvaccination samples were 12, 24, 32, and 64, respectively. These high titers may probably be regarded as an indication of a response to the vaccine.

The AN antibodies to X-31 were measured in pre- and postvaccination nasal washings from 10 subjects. A rise was observed in 7 subjects. The three who failed to show an AN antibody

increase had prevaccination titers of 4, 6, and 12, respectively.

The lymphocyte stimulation was determined in blood samples obtained on postvaccination days 0, 15, and 30 from 6 subjects. The results are shown in Table 2. All stimulation indices were <2 in all prevaccination samples and returned to the initial level on day 30. Three samples (no. 1, 15, and 17), taken on day 15, showed high levels of lymphocyte stimulation with a stimulation index varying between 5.0 and 28.5; three others showed marginally positive levels. The levels may have been influenced unfavorably by the presence of small quantities

TABLE 2. *Lymphocyte stimulation in volunteers vaccinated with "Ann" strain*

| Subject no. | SI <sup>a</sup> at postvaccination days: |      |                   | Virus dilution producing maximal stimulation (-log <sub>10</sub> ) |
|-------------|--|------|-------------------|--|
|             | 0  | 15   | 30                |  |
| 1           | <2.0 <sup>b</sup>                        | 3.0  | <2.0 <sup>b</sup> | 6  |
| 6           | <2.0                                     | 18.0 | <2.0              | 3  |
| 9           | <2.0                                     | 2.5  | <2.0              | 3  |
| 15          | <2.0                                     | 28.5 | <2.0              | 4  |
| 17          | <2.0                                     | 5.0  | <2.0              | 2  |
| 22          | <2.0                                     | 2.5  | <2.0              | 6  |

<sup>a</sup> SI, stimulation index.

<sup>b</sup> <2.0 at all virus dilution levels tested.

of thymidine in the culture medium used. Also, it is well known that stimulation by specific antigens results generally in lower stimulation levels as compared to those obtained by nonspecific stimulation (7).

## DISCUSSION

The formation of circulating antibodies against respiratory viruses must be considered as only one of the various aspects of the immune defense. Local antibodies have been shown to act as a defense barrier at the portal of entry. In addition, there is accumulating evidence that cell-mediated immunity also plays an important role in respiratory virus infections (22, 23). These two mechanisms, local antibody formation and cell-mediated immunity, may be stimulated more by the active replication of a live influenza virus in the upper respiratory tract than by the parenteral inoculation of an inactivated influenza vaccine. This may explain why, in comparative trials in man or laboratory animals, live virus vaccines given intranasally gave a better protection than inactivated vaccines administered subcutaneously (6, 18). In a recent trial a live vaccine was compared to a killed oil adjuvant vaccine, and equivalent levels of protection were obtained although the antibody titers induced by the oil adjuvant vaccine were higher (9). Circulating antibodies are, therefore, not a reliable measure of the immunity status, but, from a practical point of view, they are easier to measure.

In the present study local antibody levels were measured in pre- and postvaccination samples using the neutralization and the neuraminidase-inhibition tests. All subjects showed an increase in nasal neutralizing antibody levels; the antineuraminidase antibody levels followed a similar pattern except for two subjects where no change was observed.

Recently, the relative difficulty of producing a boosting effect in persons who already possessed antibodies was mentioned as a potential disadvantage of live influenza vaccines (2). This assumption, however, was based solely upon circulating antibody measurements (11). Our present data demonstrate that, in the presence of relatively high titers of circulating antibodies at the time of vaccination a marked booster effect of the nasal antibody titers can be obtained by the administration of a live influenza vaccine. In a given subject a significant booster of the local antibodies is not necessarily paralleled by a significant increase of the circulating antibodies. Local antibodies may be particularly desirable in the protection against influenza, because they are less specific antigenically than circulating antibodies (24).

In all our tests the IgA content of the secretions was adjusted to 200 µg/ml. In our opinion this gives a more reliable basis for comparison than titrations performed on samples without previous adjustment or based upon the total protein content.

Although there is an increasing interest in the role of cell-mediated immunity in virus infections (19), very few studies have been done on this aspect in respiratory viral infections. Waldman et al. (23) studied this phenomenon in guinea pigs and found that bronchial lymphocytes were stimulated much more after nasal inoculation than after parenteral inoculation of inactivated vaccine. He found similar results in man (22). In our trial the circulating lymphocytes were found to be stimulated in several vaccinees. The significance of this phenomenon is not known at the present time, but it may be correlated with an increased cell-mediated immunity against this specific type of influenza virus in the respiratory tract. Further studies are required, however, to determine if the stimulation observed is due to "T" lymphocytes or to "B" lymphocytes, or both. In addition, it would be of interest to study the reaction of lymphocytes from the respiratory tract. In the present trial there appeared to be a correlation between the local antibody status at the time of vaccination and the lymphocyte stimulation. The four subjects with initially low titers of local antibodies showed the highest stimulation index. The number of subjects was too small, however, to draw any definite conclusions.

In the second half of 1972, a variant of Hong Kong influenza (A/England/42/72) caused an epidemic in Australia and some other countries of the southern hemisphere and, more recently, it became widespread in the northern hemi-

sphere. In the present study, relatively high levels of antibodies against this new strain developed both in the serum and the nasal secretions of subjects having received the Ann strain. In this regard, it may be worth mentioning that inhibitor-resistant influenza strains are known to behave like antigenically "senior" strains as compared to their inhibitor-sensitive parent strains (10). Whether or not this property bears any relationship with the good response to the new strain will be an object of further study.

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