Enhanced Immunogenicity in Mice of a Purified, Tween-Ether-Treated Influenza Vaccine

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The immunogenic response of mice vaccinated intranasally or subcutaneously with increasing doses of a purified, concentrated intact A₁/Taiwan influenza vaccine or its Tween-ether derived vaccines was compared. Immunogenicity was measured by serum neutralization and hemagglutination-inhibition antibodies, lung lesions scores, and protection against respiratory challenge with live airborne influenza virus. Intact (untreated) vaccine, Tween-ether-treated (ET) vaccine, and the isolated hemagglutinins (HA) provided protection and stimulated homologous antibody response at the 35- and 70-chicken cell agglutination (CCA) unit level. At a lower dosage level, the vaccines administered by the subcutaneous route appeared to confer better protection. The ET vaccine was superior to intact virus or HA vaccines when administered subcutaneously. The minimum amount of the HA and intact vaccine given subcutaneously that protected mice against respiratory challenge was 7 CCA units (3.5 units injected twice) compared to 0.7 CCA units (0.35 units injected twice) for the ET vaccine. No heterologous antibody to the A/PR/8/34 or B/Mass/3/66 was noted. Low-level serum-neutralizing antibody was found against the A₁/Japan/170 strain but, despite high levels of homologous A₁/Taiwan/64 antibody, no cross-reactivity was found with the recent A₁/Hong Kong/68 variant.

In recent years, increased efforts have been directed toward the development of highly purified vaccines containing only the essential immunizing antigens. Davenport, Hennessy, and their associates (3, 12) have shown that purified influenza hemagglutinin (HA) obtained by ether treatment of the whole virion induced antibody against intact virus in man and animals at least equal to that obtained with whole inactivated virus. Brandon et al. (1) subsequently studied the human febrile response to intact influenza virus or purified HA obtained after ether disruption. At maximum antigen doses, ether treatment eliminated fever induction by the A/2 vaccines.

Reimer and co-workers (22) have described the purification and concentration of influenza virus preparations in a continuous flow, isopycnic-banding rotor of a K-II zonal centrifuge. Clinical evaluation of these purified vaccines (20) indicated they were equal in antigenicity to equivalent doses of conventional vaccines.

This paper summarizes experimental animal immunization studies with commercially prepared, highly purified, concentrated influenza vaccine subjected to Tween-ether treatment. Immunogenicity of the various antigen fractions was determined in mice by homotypic, heterotypic, and heterologous antibody responses, mortality rates, and evaluation of lung lesion scores.

MATERIALS AND METHODS

Experimental animals. Specific pathogen-free (SPF) male Swiss albino mice, 3 to 4 weeks old, weighing 15 to 20 g were used in the immunization and challenge studies. All mice were purchased from Manor Research, Staatsburg, N.Y. Throughout the experiments, the SPF mice were maintained in filter-topped cages and were provided with autoclaved bedding and pasteurized food and water.

Viruses. Influenza A/PR/8/34 was received from Max Rosenbaum, Naval Medical Research Unit No. 4, North Chicago, Ill.; the strains A₁/Aichi/2/68, A₁/Taiwan/1/64 (egg adapted), A₁/Japan/170/62, and B/Mass/3/66 were from E. B. Seligmann, Jr., National Institutes of Health, Bethesda, Md.; and the A₁/Taiwan/1/64 (mouse adapted) strain was from Robert Bower, Abbott Laboratories, North Chicago, Ill.

Vaccine. Chicken embryo A₁/Taiwan/1/64 vaccine (Zonomune), lot no. YV0910AMV, was purchased.
from Eli Lilly and Co., Indianapolis, Ind. This licensed, commercially obtained product is an inactivated and zonal centrifuged (22) vaccine.

**Preparation of virus fractions.** The procedure used was essentially that described by Davenport (3, 4). Two volumes of fresh, anesthetic grade ethyl ether and 1 mg of Tween 80 per ml were added to the Formalin-inactivated intact vaccine. The mixture was stirred continuously for 6 hr at 4°C. Phases were separated and the ether was discarded; excess ether was removed by bubbling with nitrogen. This process was repeated with 2 volumes of ether for 6 hr at 4°C, and a third treatment was performed with 2 volumes of ether for 1 hr at room temperature. After each treatment, phases were separated, the ether was discarded, and the aqueous phase was freed of residual ether by bubbling with nitrogen at room temperature. This fraction was designated the ether-treated (ET) material.

The HA fraction was recovered from a sample of the Tween-ether treated material by adsorption to and elution from chicken red blood cells. The supernatant representing the soluble antigen (S) fraction was adsorbed twice more or until the hemagglutination titer was <1 for two successive adsorptions.

**Determination of CCA values.** The method of Hirst and Pickels (13) was followed. Hemagglutination readings were done on intact vaccines by using a Coleman Junior Spectrophotometer (model 6C) at 542 nm. Since the chicken cell agglutination (CCA) test has questionable value with ether-treated influenza virus preparation (4), the HA and ET antigens were standardized by dilution to the same degree as the starting intact preparations (1, 11).

**Hemagglutination and HI titrations.** Tests were performed in duplicate by the microtiter method (25) in disposable "U" plates (Cooke Engineering Co., Alexandria, Va.) as described by Davenport and Minuse (5). In all tests, 1% chicken red blood cells were used; in the hemagglutination-inhibition (HI) test, four hemagglutinating units of antigen were used, all antiserums were heated at 56°C for 30 min and trypsin-periodate treated to remove nonspecific inhibitors of hemagglutination.

**SN test.** The protocol used for serum-virus neutralization (SN) test was similar to that described in the USPHS Requirements (6). Sera were heat inactivated at 56°C for 30 min, serially diluted, and incubated at 4°C for 1 hr with an equal volume of 10 to 320 LD₅₀ (21) of various strains of influenza virus. This serum-virus mixture was then tested in mice or embryonated eggs.

Each mouse was anesthetized with ether, given an intranasal instillation of 0.05 ml of the mixture, and observed for 10 to 14 days; the deaths were then recorded. As a control, normal mouse serum mixed with the virus and normal serum alone were administered to mice.

Ten-day-old embryonated chicken eggs were inoculated by the allantoic route with 0.1 ml of virus-serum mixture, incubated at 37°C for 48 hr, and harvested when an EID₉₀ dose of 32 to 320 was attained as indicated by hemagglutination of the virus control. The EID₉₀ dose was determined by parallel infectivity tests in eggs by using a 0.1-ml virus-saline mixture. As controls, phosphate-buffered saline (PBS) only, PBS plus normal mouse serum, and normal mouse serum plus virus were inoculated into eggs.

**Aerosol challenge.** Aerosol challenge studies were conducted in a 300-liter plastic chamber (60 by 95 cm) installed within a microbiological safety cabinet. A University of Chicago Toxicity Laboratory type atomizer was used to produce airborne particles of 1- to 5-μm mass medium diameter (MMD). The virus was fed to the atomizer by a 50-ml syringe activated with a motor-driven piston delivering 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of approximately 33 liters/min. The humidity in the chamber was maintained at 72 to 84% relative humidity. The aerosol chamber was sampled with a standard all-glass impinger (AGI-30) employing PBS with 0.2% bovine serum albumin (BSA) as a collecting fluid. The inhaled dose of virus was determined by the method of Rosebury (23), which is based upon the product of the aerosol concentration, minute volume of respiration, and the duration of exposure.

For challenge studies, groups of eight vaccinated mice were placed in the aerosol chamber and exposed for 10 to 15 min to either mouse-adapted influenza A₂/Taiwan or A/PR/8 virus. The mice, air washed for 10 min prior to removal from the aerosol chamber, were then held for 14 days in an isolated animal room protected from airborne or cross-infection by filter-capped cages. As a control for these studies, nonvaccinated mice were challenged with either aerosolized 0.2% BSA or influenza virus.

**Scoring of pulmonary lesions.** The extent of pulmonary lesions was expressed as a percentage of the total lung consolidated (14). A score of 1 represented 25% lung consolidation; 2 = 50%, 3 = 75%, 4 = 100%, and 5 represented death.

**Experimental protocol.** Groups of eight mice were vaccinated by either the subcutaneous (sc) or intranasal (in) route with graded doses ranging from 0.35 to 310 CCA units of either intact (untreated), ET, or HA vaccines. Since the S fraction contained no demonstrable HA, it was diluted with PBS to obtain concentrations equivalent to the above vaccines. A 1:1,000 dilution was, therefore, comparable to 0.35 CCA units. All vaccines and the saline control contained 100 units of polymyxin B and 100 μg of neomycin per ml. The sc dose was contained in a 0.2-ml volume and was injected into the upper thoracic region between the scapulae. For the in dose, each mouse was anesthetized with ether and a total volume of 0.05 ml of vaccine was instilled in both nares.

Three weeks after vaccination, groups of eight mice were revaccinated with either saline or the same dilution of antigen as given originally. One week after the secondary vaccination, half of the mice were bled and the remaining were challenged with airborne influenza virus. After the infectious challenge, mice were observed for 14 days with all surviving mice sacrificed for lung lesion scoring.
RESULTS

Infectivity of airborne influenza virus. To obtain maximal infectivity, influenza A5/Taiwan/1/64 virus used for the infectious challenge study was passaged intranasally five times in mice. Preliminary aerosolization infectivity studies indicated that A/PR/8 virus suspended in PBS with 0.2% BSA as a stabilizer was more infective for mice than when suspended in PBS alone or in Tryptose Phosphate Broth (Table 1). This diluent was therefore used for the aerosolization experiments employing A/PR/8 and A5/Taiwan viruses.

Comparative neutralization tests in mice and eggs. Selected sera obtained from mice vaccinated sc with the various influenza vaccines were tested initially in both mice and eggs for virus neutralization. The results shown in Table 2 indicate that the SN tests were comparable in both assay systems. Since neutralization tests in eggs were more rapid and less expensive than in mice, further evaluation of SN titers was done only in eggs.

Antigenicity studies. The serologic response and respiratory challenge data from mice vaccinated in or sc with the various vaccines are summarized in Tables 3 and 4. The Hirst titration on the intact vaccine gave a value of approximately 775 CCA units/ml. Therefore, the maximum antigenic dose given in was 35 CCA units/0.05 ml injection.

Vaccines diluted to 0.35 CCA units were in most cases not immunogenic. However, two injections of the ET vaccine given by the sc route (Table 4) were effective when measured by decrease in mortality and lung lesion scores. With this exception, the inconsistent mortality data obtained with 0.35 CCA units of the various vaccines given sc or in cannot be explained. For example a higher mortality rate occurred in mice given two sc injections (0.70 CCA units) of the HA fraction than in those given primary HA followed by a saline injection (Table 4). In Table 3, the data indicate that two injections of the ET given in are less immunogenic than one injection followed by saline. The marginal amount of antigenic mass (0.35 CCA units) may account for some of these inconsistencies.

When 3.5 CCA units of the vaccines was administered in or sc minimal or no antibody response was noted. However, the effectiveness of the various vaccines was still demonstrable by decreased mortality rates and lung lesion scores. This was particularly obvious with mice given two subcutaneous injections of intact, ET, and HA vaccines (Table 4). Similar results were not found when these vaccines were given by the in route (Table 3). Only the HA fraction given twice or the intact vaccine given once provided any protection.

The 15.5- and 31-CCA unit vaccines were administered only by the sc route (Table 4). After one injection of the 15.5-CCA vaccines, only the ET vaccine stimulated homologous antibody and that was very low (1:5 SN titer). After exposure to airborne A5/Taiwan virus, the intact- and ET-vaccinate groups had a decreased mortality rate and lung lesion score. The HA-vaccinate group was not protected.

An anamnestic response was evident after mice were given two injections of the intact or

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**TABLE 2. Comparative mouse serum neutralization tests in mice and eggs using A5/Taiwan virus**

| Subcutaneous vaccination (35 CCA units) | Reciprocal homologous serum titers, neutralization test in Mice (60 to 100 MNLD_{50}) | Egg (32 to 44 EID_{50}) |
|----------------------------------------|-------------------------------------------|------------------------|
| Primary                               |                                          |                        |
| Intact                                | 11                                       | 6                      |
| ET                                    | 32                                       | 11                     |
| HA                                    | <4                                       | <4                     |
| S                                     | 4                                        | <4                     |
| Saline                                | <2                                       | >2                     |
| Secondary                             |                                          |                        |
| Intact-intact                         | 90                                       | 91                     |
| Intact-saline                         | 5                                        | 14                     |
| ET-ET                                 | 305                                      | 324                    |
| ET-saline                             | 5                                        | <4                     |
| HA-HA                                 | 147                                      | 91                     |
| HA-saline                             | <4                                       | 11                     |
| S-S                                   | 28                                       | 64                     |
| S-saline                              | <4                                       | <4                     |
| Saline-saline                         | <2                                       | >2                     |

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**TABLE 1. Infectivity of airborne A/PR/8 virus in various diluents**

| Virus dilution | Diluent<br> | TPB | PBS | PBS + 0.2% BSA |
|----------------|-------------|-----|-----|----------------|
| 10^{-1}        | 3/4<sup>a</sup> | 4/4 | 4/4 |
| 10^{-2}        | 0/4         | 0/4 | 3/4 |
| 10^{-3}        | 0/4         | 0/4 | 1/4 |
| Diluent only   | 0/4         | 0/4 | 0/4 |

<sup>a</sup> Mice were exposed to aerosolized virus for 5 min at 80 to 83% relative humidity. All diluents contained 100 units of polymyxin B sulfate and 100 μg of neomycin sulfate per ml.

<sup>b</sup> TPB, Tryptose Phosphate Broth (Difco); PBS, phosphate-buffered saline; BSA, bovine serum albumin.

<sup>e</sup> Dead/total.
TABLE 3. Serological and respiratory challenge data obtained from mice vaccinated intranasally with various ether-treated A2/Taiwan fractions

| No. of injections of vaccine | Total CCA units injected | Reciprocal homologous titers | Respiratory challenge | % per cent mortality | Average lung lesion |
|-----------------------------|--------------------------|-------------------------------|-----------------------|---------------------|--------------------|
|                             | SNb HI                    | Vaccinated                    | Control               | Vaccinated          | Control            |
| Intact vaccine              |                          |                               |                       |                     |                    |
| 1                           | 0.35 <4 <8               | 86                            | 100.4.86              | 5.00                |                    |
| 2                           | 0.70 <4 <8               | 43                            | 100.3.57              | 5.00                |                    |
| 1                           | 3.5 <4 <8                | 38                            | 100.2.75              | 5.00<4              |                    |
| 2                           | 7.0 >16 8                | 63                            | 100.3.63              | 5.00                |                    |
| 1                           | 35 <4 <8                 | 0                             | 63.1.25               | 4.50<4              |                    |
| 2                           | 70 53 32                 | 13                            | 63.0.63               | 4.50<4              |                    |
| ET vaccine                  |                          |                               |                       |                     |                    |
| 1                           | 0.35 6 <8                | 43                            | 100.2.86              | 5.00<4              |                    |
| 2                           | 0.70 <4 <8               | 83                            | 100.4.83              | 5.00                |                    |
| 1                           | 3.5 <4 <8                | 67                            | 100.4.00              | 5.00                |                    |
| 2                           | 7.0 <4 <8                | 83                            | 100.4.83              | 5.00                |                    |
| 1                           | 35 6 <8                  | 0                             | 63.1.13               | 4.50<4              |                    |
| 2                           | 70 205 64                | 13                            | 63.1.00               | 4.50<4              |                    |
| HA vaccine                  |                          |                               |                       |                     |                    |
| 1                           | 0.35 <4 <8               | 88                            | 100.4.38              | 5.00                |                    |
| 2                           | 0.70 6 <8                | 86                            | 100.4.43              | 5.00                |                    |
| 1                           | 3.5 <4 <8                | 88                            | 100.4.50              | 5.00                |                    |
| 2                           | 7.0 <4 <8                | 86                            | 100.3.63              | 5.00                |                    |
| 1                           | 35 <4 <8                 | 0                             | 63.1.63               | 4.50<4              |                    |
| 2                           | 70 45 16                 | 13                            | 63.0.63               | 4.50<4              |                    |

a Groups of eight mice were challenged with 0.63 to 31.5 MINLD_{50} of airborne A2/Taiwan influenza virus.
b EID_{40} dose of virus used in neutralization test varied from 32 to 44.
c Three weeks after the first injection of A2/Taiwan vaccines, mice were given a second injection of the same vaccine or saline. All animals were bled 1 week later.
d Significant difference between vaccinated mice and corresponding controls at P < 0.05, as determined by the t-test.

ET 15.5-CCA unit vaccines. Both SN and HI titers reflected this response. Even though two injections of the HA fraction stimulated very low SN antibody and no measurable HI antibody, the decreases in mortality and lung lesion score were comparable to those observed in animals given two injections of intact and ET vaccines.

HI antibody was not detected in mice given a primary sc or in injection of 35 CCA units of all A2/Taiwan vaccines (Table 3, 4). However, low levels of neutralizing antibody were noted in some cases and decreased mortality rates and lung lesion scores were found in all cases but the intact fraction given sc. When mice were given a second injection of the 35-CCA unit vaccines (70 CCA units), a strong anamnestic response, as measured by both HI and neutralizing anti-

TABLE 4. Serological and respiratory challenge data obtained from mice vaccinated subcutaneously with various ether-treated A2/Taiwan fractions

| No. of injections of vaccine | Total CCA units injected | Reciprocal homologous titers | Respiratory challenge | % per cent mortality | Average lung lesion |
|-----------------------------|--------------------------|-------------------------------|-----------------------|---------------------|--------------------|
|                             | SNb HI                    | Vaccinated                    | Control               | Vaccinated          | Control            |
| Intact vaccine              |                          |                               |                       |                     |                    |
| 1                           | 0.35 <4 <8               | 63                            | 100.4.25              | 3.88                |                    |
| 2                           | 0.70 <4 <8               | 71                            | 100.3.71              | 3.88                |                    |
| 1                           | 3.5 <4 <8                | 25                            | 100.2.88              | 4.13                |                    |
| 2                           | 7.0 10 <8                | 0                             | 63.1.63               | 4.13<4              |                    |
| 1                           | 15.5 <4 <8               | 25                            | 75.2.38               | 4.50<4              |                    |
| 2                           | 31 56 48                 | 25                            | 75.2.38               | 4.50<4              |                    |
| 1                           | 35 14 <8                 | 50                            | 63.3.00               | 3.88<4              |                    |
| 2                           | 70 91 32                 | 0                             | 63.7.35               | 3.88<4              |                    |
| 1                           | 155 121 48               | 0                             | 100.0.50              | 5.00<4              |                    |
| 2                           | 310 192 96               | 0                             | 100.0.00              | 5.00<4              |                    |
| ET vaccine                  |                          |                               |                       |                     |                    |
| 1                           | 0.35 <4 <8               | 25                            | 100.2.50              | 3.88                |                    |
| 2                           | 0.70 4 <8                | 13                            | 100.3.63              | 3.88<4              |                    |
| 1                           | 3.5 <4 <8                | 25                            | 100.2.25              | 4.13<4              |                    |
| 2                           | 7.0 <4 <8                | 13                            | 63.1.30               | 4.13<4              |                    |
| 1                           | 15.5 <4 <8               | 22                            | 75.1.89               | 4.50<4              |                    |
| 2                           | 31 60 24                 | 0                             | 75.0.38               | 4.50<4              |                    |
| 1                           | 35 <4 <8                 | 13                            | 63.1.38               | 3.88<4              |                    |
| 2                           | 70 324 128               | 0                             | 63.1.38               | 3.88<4              |                    |
| 1                           | 155 45 16                | 0                             | 100.0.88              | 5.00<4              |                    |
| 2                           | 310 543 96               | 0                             | 100.0.00              | 5.00<4              |                    |
| HA vaccine                  |                          |                               |                       |                     |                    |
| 1                           | 0.35 4 <8                | 13                            | 63.2.63               | 3.88                |                    |
| 2                           | 0.70 <4 <8               | 75                            | 63.4.25               | 3.88                |                    |
| 1                           | 3.5 <4 <8                | 63                            | 63.6.33               | 4.13                |                    |
| 2                           | 7.0 8 <8                 | 13                            | 63.6.75               | 4.13<4              |                    |
| 1                           | 15.5 <4 <8               | 100                           | 88.5.00               | 4.63                |                    |
| 2                           | 31 5 <8                  | 25                            | 88.1.88               | 4.63<4              |                    |
| 1                           | 35 11 <8                 | 25                            | 63.2.50               | 3.88                |                    |
| 2                           | 70 91 64                 | 0                             | 63.6.33               | 3.88<4              |                    |
| 1                           | 155 25 8                 | 0                             | 75.0.63               | 4.00<4              |                    |
| 2                           | 310 768 96               | 0                             | 75.1.00               | 4.00<4              |                    |

a Groups of eight mice were challenged with 0.63 to 31.5 MINLD_{50} of airborne A2/Taiwan influenza virus.
b EID_{40} dose of virus used in neutralization test varied from 10 to 46.
c Three weeks after the first injection of A2/Taiwan vaccines, mice were given a second injection of the same vaccine or saline. All animals were bled 1 week later.
d Significant difference between vaccinated mice and corresponding controls at P < 0.05 as determined by the t-test.
bodies, was found with all fractions. The serological data correlated well with the decreased mortality and lung lesion scores. The greatest antibody response occurred in mice given the ET vaccine. The antigenic mass of the 35-CCA vaccines, therefore, administered by in or sc routes, gave comparable results and were sufficient to stimulate antibody, reduce mortality, and decrease lung lesion scores.

Sera from mice injected with the various fractions of 35-CCA unit vaccines were also tested for neutralizing and HI antibody against influenza A2/Aichi/2/68, B/Mass/3/66, A/PR/8, and A2/Japan/170/62. There were no detectable neutralizing antibodies to the A2/Aichi, B/Mass, or A/PR/8 virus strains. Serum from mice vaccinated twice with the ET fraction by the in or sc route had SN titers of 1:23 and 1:10, respectively, against 78 EID50 A2/Japan/170 virus. HI tests with these four influenza strains showed no cross-reactions when the mouse sera were tested at an initial 1:8 dilution.

In the study using only sc injections of 155 and 310 CCA units of the various A2/Taiwan antigens, mice were challenged with aerosolized homologous as well as A/PR/8 virus. In mice given a primary sc injection of 155 CCA units of intact, ET, and HA vaccines, both HI and SN homologous antibody was detected (Table 4). However, no HI (< 8) or SN (< 4) antibody was noted against A/PR/8, A2/Aichi, and B/Mass viruses. After challenge with aerosolized A2/Taiwan virus, a decrease in lung lesion scores was found in the intact-, ET-, and HA-vaccinated groups. No protection was found when animals were given aerosolized A/PR/8 virus.

After a second sc injection of the various fractions, an anamnestic response was noted with the ET and HA vaccines, as measured by HI or SN titers, or both. Even though high titers were found with two injections of intact vaccine, they were not significantly higher than after one injection (SN titer of 1:121 versus 1:192 and HI titer of 1:48 versus 1:96). The effectiveness of these homologous titers was reflected in a decreased mortality rate and average lung score in mice challenged with the airborne A2/Taiwan virus. Despite the excellent homologous antibody response, no heterologous HI (<8) and SN (<4) antibodies were detected against A/PR/8, A2/Aichi, or B/Mass influenza viruses. In addition, after vaccinated animals were exposed to airborne A/PR/8 virus, the mortality rate and lung lesion scores were as high as in the nonvaccinated animals.

Inconsistent results were obtained with the S vaccine administered by the sc and in routes. As measured by reduced mortality rates and lung lesion scores, the 1:1,000 dilution was protective when given as a primary but not as a secondary vaccination. The 1:100 vaccine was not immunogenic when administered by either route. Two sc injections of a 1:10 dilution resulted in both SN (1:64) and HI (1:16) antibody responses as well as reduced mortality and lung lesion scores. In addition, two in injections of the 1:10 dilution, as well as two sc injections of the undiluted S vaccine, produced low levels of SN antibody (1:11 and 1:20, respectively) and protection against respiratory challenge without demonstrable HI antibody.

**DISCUSSION**

Our studies demonstrated that the immunogenic response appeared to be independent of the route of vaccination at the 35- and 70-CCA dose of the intact A2/Taiwan, ET, and HA vaccines. At a lower dosage level, the vaccines administered by the subcutaneous route appeared to confer better protection. A dose-response relationship was seen with all three vaccines. That is, the greater the antigenic mass, the higher the antibody titers and the greater the protection against respiratory challenge with homologous A2/Taiwan virus. Similar findings with intact influenza vaccines have been reported (7, 15).

Fazekas de St. Groth has suggested that antibody present in the respiratory tract secretions may play an important role in protection against reinfection with respiratory viruses (8). He and Donnelly (9) showed that antibody in respiratory secretions was primarily responsible for protection against experimental influenza in mice. Similarly, Francis (10), after administering inactivated influenza vaccines, demonstrated virus neutralizing activity in nasal secretions. Although nasal secretion antibody was not measured in our study, the data strongly suggest that a mechanism other than serum antibody was also present in the vaccinated mice. In many cases there was no correlation between serum antibody titers and protection against airborne influenza virus (Table 4). This was particularly true in those animals vaccinated sc with minimal amounts of antigen. Since the challenge with live virus was 7 to 28 days after vaccination, it is reasonable to assume that interferon played little or no role in the protection.

Kaye and co-workers (16) found a direct relationship between HI antibody and protection against infection in mice. In our study, this was generally true only in those animals given higher vaccine doses (≥ 31 CCA units). When smaller antigenic doses (< 31 CCA units) were given sc, protection against respiratory challenge was
frequently observed although HI and most SN titers were < 8.

The effect of serial sc injections on antibody response and protection in mice was also observed (Table 4). For example, 31 CCA units of intact vaccine, given in two doses of 15.5 units each, stimulated SN and HI antibody levels 4- and 12-fold higher, respectively, than when the 35 CCA units were given as single injection. In addition, the two injections resulted in a considerable decrease in mortality and a significant decrease in lung lesion scores. Similarly, 15.5 units of ET vaccine given twice (31 units total) gave SN and HI antibody titers 30- and 6-fold higher, respectively, than when a 35-unit ET vaccine was given once. Protection was noted in all cases. The data thus indicate that any correlation of serological response and protection should be based in part on the number of injections of antigen. Meiklejohn (18) reported that, in human subjects, the same number of CCA units of A2 intact vaccine divided into two injections produced a response better than that obtained with a single injection.

None of the vaccines stimulated heterologous antibody against influenza A/PR/8/34 or B/Mass/3/66. In addition, no protection was noted when vaccinated mice were challenged with airborne A/PR/8 virus. Low serum-neutralizing antibody titers were noted against the A2/Japan/170 strain. Although homologous A2/Taiwan-neutralizing antibody titers were as high as 1:768, no SN and HI antibodies were detected against the A2/Aichi/2/68 strain, which is representative of the new A2/Hong Kong/68 variant. The absence of cross-reactivity between the A2/Taiwan and A2/Aichi strains has been reported by Brown et al. by using human sera (2).

The intact, HA, and ET vaccines provided protection and stimulated antibody response at the ≥ 70-CCA unit level. The minimum amount of the HA and intact fractions that protected mice after sc vaccination was 7.0 CCA units (3.5 units given twice), as compared to 0.7 CCA units (0.35 units given twice) for the ET vaccine (Table 4). The HA and purified, concentrated intact vaccines were comparable in their immunogenicity, confirming reports that these two vaccines induced equivalent HI and SN antibodies in humans (3, 12). The ET vaccine, which had been Tween-ether treated only, was superior to both the HA and intact vaccines.

Neurath et al. (19) reported that Tween-ether treatment released neuraminidase from the HA of three A2 influenza viruses, including A2/Taiwan/66. Recent studies by Schulman et al. (24) showed that antibody directed against neuraminidase inhibited influenza A2 virus replication in the lungs of infected mice even in the absence of HI antibody. Although neuraminidase antibody was not measured in our study, its role in the enhanced immunogenicity of the ET vaccine should be considered. Immunogenicity of the S fraction, also reported by Lange (17), could possibly be attributed to neuraminidase released after ether treatment. However, contamination of the S fraction with undetected monomeric HA may have occurred.

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