INFECTION AND IMMUNITY, Dec. 1974, p. 1329-1336

Vol. 10, No. 6
Printed in U.S.A.

# Inactivated Influenza Vaccine Efficacy: Diminished Antigenicity of Split-Product Vaccines in Mice

DAVID W. BARRY, ELDRIDGE STATON, AND RONALD E. MAYNER
*Food and Drug Administration, Bethesda, Maryland 20014*

Received for publication 28 June 1974

Groups of 60 to 120 mice were given a single intraperitoneal inoculation of varying dilutions of commercially prepared and licensed bivalent (A/England and B/Mass) and monovalent (A/Aichi or B/Hong Kong) inactivated influenza vaccines, and their antibody responses at 14 days were quantitated by hemagglutination inhibition tests. Split-product vaccines prepared by the treatment of A/England, B/Mass, and B/Hong Kong whole virus with Tween-80 and either tributylphosphate or ether produced significantly lower mean antibody titers than did equivalent whole-virus preparations. The rates of seroconversion ($<$1:8 to $\geq$1:8) at the various dilutions tested were also significantly reduced when these split-product vaccines were given. When the antigen content of all vaccines was quantitated by the chick cell agglutination test, between 10 and 100 times more split-product antigen than whole-virus antigen was required to produce seroconversion in 50% of the mice tested. Differences between split-product and whole-virus A/Aichi vaccines were less marked. These data point out the need to consider factors other than hemagglutinin content alone in determining the immunogenicity of inactivated influenza vaccines.

Influenza virus infection continues to be a major cause of morbidity in the United States and is the only infectious disease that regularly produces significant excess mortality (34) in spite of the long-standing availability and annual use of 15 to 25 million doses of an effective vaccine. Although the cause of this apparent paradox has in part been related to inadequate utilization of the vaccine in high-risk individuals (34), concern over possible variations in the potency and efficacy of influenza vaccines has prompted us to re-examine the relationship between the laboratory and clinical indexes of the protective capacity of modern, highly purified, and commercially available influenza vaccines. The following report describes the results of the first of a series of studies undertaken to investigate various in vitro and in vivo parameters of the potency of these vaccines.

Because influenza virus vaccine, unlike most other contemporary viral vaccines, is an inactivated product, indirect methods of potency quantitation must be used. Currently, the potency of these vaccines is measured by the chick cell agglutination (CCA) test (20), which directly quantitates only the hemagglutinin component of the virus. Although the test is relatively precise and reproducible when values obtained for a given vaccine are compared with a standard reference (37), previous experiments have shown that differing egg passages (19) and various manufacturing procedures (5, 12, 23, 31, 35) might alter the relationship between the immunogenicity of influenza vaccines and their CCA content. Techniques that quantitate the immunogenic potential of vaccines in experimental animals by antigen extinction methods have therefore been suggested (27, 35) as more relevant in determining the protective capacity of inactivated vaccines. Previous two-step antigen extinction techniques involving the intranasal instillation of pooled immune serum and virus mixtures into mice (7) have been shown to be cumbersome, poorly reproducible (13, 37), and excessively dependent on the virulence of the mouse-adapted challenge virus (26). Since serum hemagglutination inhibiting (HI) antibodies produced in mice after parenteral inoculation of inactivated influenza vaccines are well correlated with protection from infection (10, 17), we have evaluated the immunogenicity of a number of the commercially produced monovalent and bivalent influenza vaccines available for human use by quantitating the antibodies they produce in mice.

Two of the vaccine types tested are manufactured by treatment of the whole virus with Tween-80 and either ether or tributylphosphate (TBP) in an effort to diminish the local and systemic reactions produced by influenza vac-

cines (18). Previous experiments on the protective efficacy of experimentally produced "split-product" influenza vaccines in mice were somewhat contradictory (5, 12, 23), whereas earlier experience with inactivated measles vaccines subjected to ether treatment had shown them to be, in some respects, less immunogenic than equivalent whole-virus vaccines (24, 25). This investigation was designed, therefore, to elucidate any differences that may exist between the immunogenic capacity, in large groups of mice, of whole-virus and split-product influenza vaccines.

## MATERIALS AND METHODS

**Mice.** Six-week-old male NIH general-purpose Swiss albino mice weighing 24 to 28 g were obtained from the NIH Rodent and Rabbit Production Section and housed 10 per cage.

**Vaccines.** Monovalent vaccines made from A/Aichi prototype virus were obtained by special contract from the six manufacturers of influenza vaccines. Bivalent vaccines containing A/England and B/Mass prototypes and monovalent vaccines made from a B/Hong Kong prototype were obtained from commercial distributors. Specific strains used in the vaccines are shown in Table 1. Both the bivalent and the monovalent B/Hong Kong vaccines had passed Food and Drug Administration minimal requirements for potency and were in general clinical use at the time of the study. The types of vaccines and the CCA values obtained for each are included in Table 2.

**CCA tests.** CCA determinations were performed as previously described (20) and modified (37) to include a standard reference. Vaccine-to-reference ratios thus obtained were multiplied by the assigned value of the 1972 reference (1,886 CCA units/ml) and the results were expressed as CCA units per human dose (0.5 ml). Multiple determinations were performed on each vaccine, and the coefficient of error approximated 14%. Accurate determinations could not be performed on vaccine Fa because of the presence of $AlPO_4$, and its assigned CCA value was that obtained for the same lot of vaccine before $AlPO_4$ was added. Since the A and B components of the bivalent vaccines were formulated at a ratio of 7:3, 70% of the total CCA value obtained for an individual bivalent vaccine was assigned to the A/England component and 30% was assigned to the B/Mass component.

**Animal inoculations.** Groups of 20 mice per dilution were individually inoculated intraperitoneally with 0.5 ml of a $10^{-1}$, $10^{-2}$, or $10^{-3}$ dilution of stock vaccine. In any individual test, both a whole-virus and a split-product vaccine were titrated simultaneously. In addition, 10 mice per test were inoculated with only the phosphate-buffered saline diluent (pH 7.4) to serve as controls. All bivalent vaccines were titrated in two separate mouse antigen extinction tests, as were four of the seven monovalent A/Aichi vaccines. Fourteen days after the single inoculation, when antibody response has been shown to be maximal (9), all mice were exsanguinated by severing the axillary artery. Individual sera were collected, treated with *Vibrio cholerae* neuraminidase, heated at 56 C for 30 min, and incubated with chick erythrocytes to remove nonspecific inhibitors (38) before being used in the HI tests.

**Serological testing.** Individual mouse sera were tested in duplicate by the HI procedure as modified for microtitration plates (36) on an automatic diluting and dispensing apparatus (Dynatiter; Cooke Engineering Co., Alexandria, Va.). The serum was tested in twofold dilutions starting at 1:8, and 4 to 8 units of homotypic whole-virus antigen was added to each well.

TABLE 2. *CCA content of vaccines tested*

| Manufacturer | Type of vaccine[a] | A/England | B/Mass | B/HK | A/Aichi |
|---|---|---|---|---|---|
| A | ZPWV | 693 | 297 | 610 | 770 |
| B | ZPWV | 805 | 385 | 620 | 650 |
| C | ZPWV | 1,008 | 432 | 575 | 1,060 |
| D | CPWV | 735 | 315 | | 1,100 |
| E | TBP | 784 | 336 | 785 | 900 |
| F | ET | 868 | 372 | 576 | 971 |

[a] Abbreviations: ZPWV, zonally purified whole virus; CPWV, chromatographically purified whole virus; TBP, tributylphosphate-treated split product; ET, ether-treated split product.

TABLE 1. *Strains used in vaccines*

| Manufacturer | Prototype | | | |
|---|---|---|---|---|
| | A/England | B/Mass | B/Hong Kong | A/Aichi |
| A | A/England/42/72 | B/Mass/1/71 | B/HK/5/72 | A/Aichi/2/68 |
| B | A/Victoria/4/72 | B/Mass/1/71 | B/HK/5/72 | X-31[a] |
| C | X-37[b] | B/Mass/1/71 | BX-1[c] | A/Aichi/2/68 |
| D | X-37A[d] | B/Mass/1/71 | | X-31 |
| E | X-37 | B/Mass/1/71 | B/HK/5/72 | A/Aichi/2/68 |
| F | X-37 | B/Mass/1/71 | B/HK/5/72 | X-31 |

[a] High-growth recombinant ($H_3N_2$) produced from A/Aichi/2/68 X A/PR/8.
[b] High-growth recombinant ($H_3N_2$) produced from A/England/42/72 X A/PR/8.
[c] High-growth recombinant ($H_3N_2$) produced from MRC-7 (A/England isolated in eggs X A/PR/8) X A/PR/8.
[d] High-growth recombinant produced from B/Hong Kong/5/72 X B/Lee/40.

**Statistics.** Fifty percent seroconversion (<1:8 → ≥1:8) rates were determined by the method of Karber. Differences in these rates were determined by the chi-square test using the Yates correction. The *t* test was used for the comparison of mean antibody titers.

## RESULTS

Geometric mean antibody titers produced in mice by the various vaccines are shown in Fig. 1 through 4. Split-product bivalent vaccines (E+F) formulated from A/England and B/Mass prototypes were distinctly less immunogenic at all dilutions than equivalent whole-virus vaccines (A–D) of approximately the same CCA value (Fig. 1 and 2). The lowest geometric mean titer of antibody produced by a whole-virus vaccine was greater, at the 0.001 level of significance, than the highest geometric mean titer induced by a split-product vaccine at all dilutions tested. The whole-virus preparations containing the least amount of antigen as determined by CCA tests (stock vaccine diluted $10^{-3}$) induced more HI antibody than either of the split-product vaccines containing approximately 100 times as much antigen (stock vaccine diluted $10^{-1}$). When monovalent vaccines prepared from B/Hong Kong prototypes are considered (Fig. 3), the immunogenic inferiority in mice of split-product vaccines remains evident even though higher concentrations (vaccines diluted $10^{-1}$) of such vaccines produced levels of antibody equal to or slightly greater than those produced by dilutions ($10^{-2}$ or $10^{-3}$) of whole-virus vaccines containing 10 to 100 times less CCA antigen. At each dilution, however, the whole-virus vaccines produced significantly more HI antibody than did the split-product vaccines. In the case of vaccines formulated with A/Aichi prototype strains (Fig. 4), the least immunogenic whole-virus vaccine (C) was significantly superior only to the ether-treated (ET) split-product vaccine (F) and only at the $10^{-1}$ dilution. Addition of $AlPO_4$ to the ET preparation markedly enhanced its immunogenicity so that it was significantly superior to the best whole-virus preparation at the $10^{-1}$ dilution and significantly superior to the ET vaccine from which it was derived at the $10^{-1}$ and $10^{-2}$ dilutions.

Seroconversion rates for the different vaccines and antigens are summarized in Fig. 5 through 8. The differences between whole and

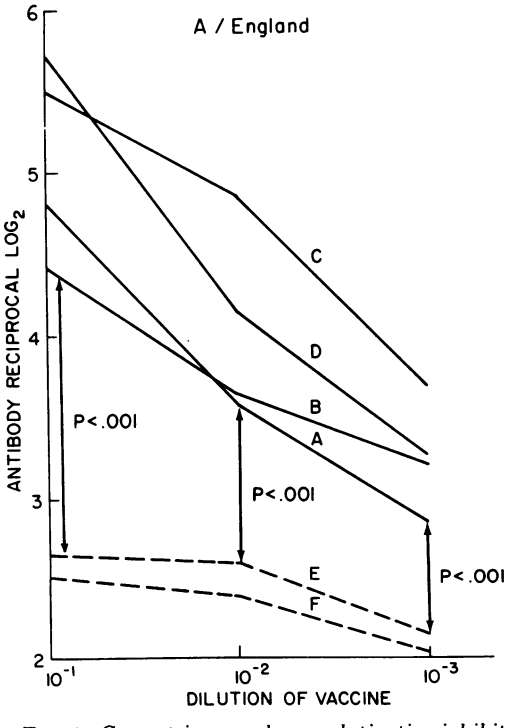


FIG. 1. *Geometric mean hemagglutination inhibiting antibody response to A/England antigen at differing dilutions of vaccine. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines.*

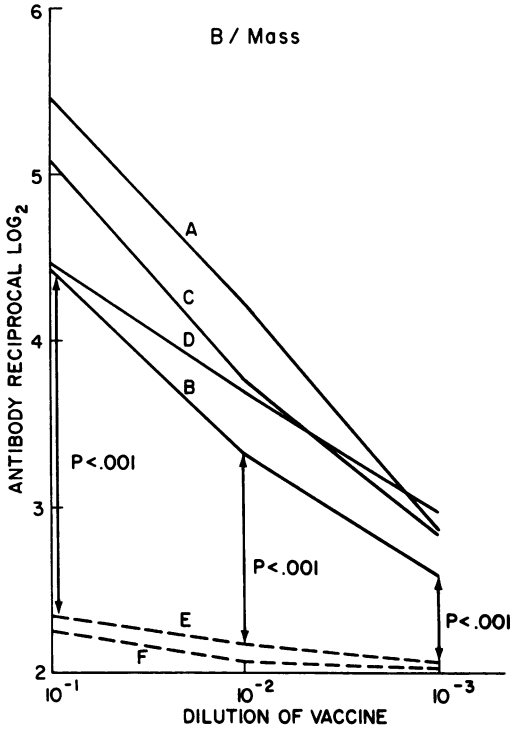


FIG. 2. *Geometric mean hemagglutination inhibiting antibody response to B/Mass antigen at differing dilutions of vaccine. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines.*

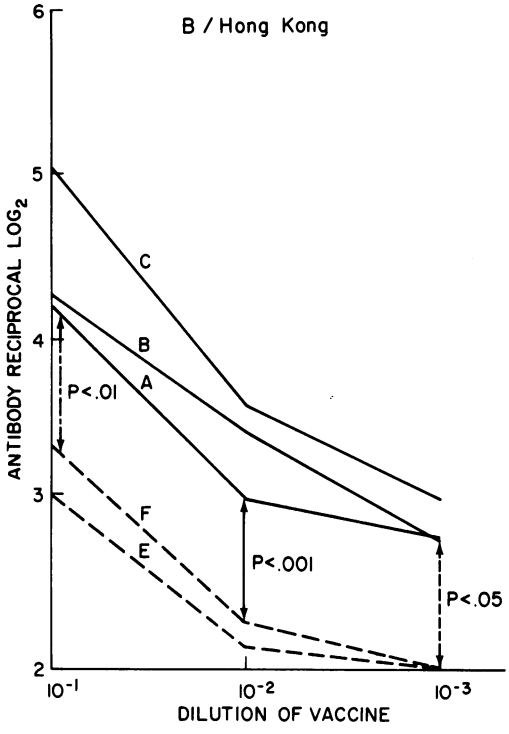


FIG. 3. *Geometric mean hemagglutination inhibiting antibody response to B/Hong Kong antigen at differing dilutions of vaccine. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines.*

split-product vaccines generally paralleled those observed when mean antibody titers were considered. Seroconversion rates produced by the ET split-product A/England vaccine were significantly lower than the lowest rate produced by any whole-virus vaccine at any of the three dilutions tested (Fig. 5). The seroconversion rates produced by the TBP-treated A/England vaccine, although lower than those produced by any of the whole-virus vaccines, were significantly lower than the lowest whole-virus rate only at the $10^{-3}$ dilution. In the B/Mass component of the bivalent preparation, however, both split-product preparations induced antibody conversion rates significantly lower than the lowest whole-virus rate at all dilutions (Fig. 6). The lowest rate produced by a whole-virus B/Hong Kong vaccine was significantly higher than that produced by the TBP-treated vaccine at the $10^{-2}$ dilution and by both split-product vaccines at the $10^{-3}$ dilution (Fig. 7). Although the seroconversion rates produced by the split-product A/Aichi vaccines are diminished, they are not significantly lower than the lowest rate produced by a whole-virus vaccine. The addition of $AlPO_4$ to the ether-treated preparation increased its capacity to induce higher rates of seroconversion at all dilutions (Fig. 7).

To adjust for differences in the CCA content of each vaccine, a 50% minimal immunizing dose was calculated by dividing the arithmetic expression of the dilution of vaccine that produced 50% seroconversion into the CCA content of the undiluted vaccine. These values, expressed in terms of CCA units required to produce 50% seroconversion in mice, are delineated in Table 3. The differences between the split-product and whole-virus vaccines are apparent when either the bivalent A/England-B/Mass or the monovalent B/Hong Kong vaccines are considered. Between less than 0.73 to 1.68 CCA units of whole-virus A/England and 0.31 to 1.50 CCA units of whole-virus B/Mass were required to seroconvert 50% of the mice tested, but over 78 and 34

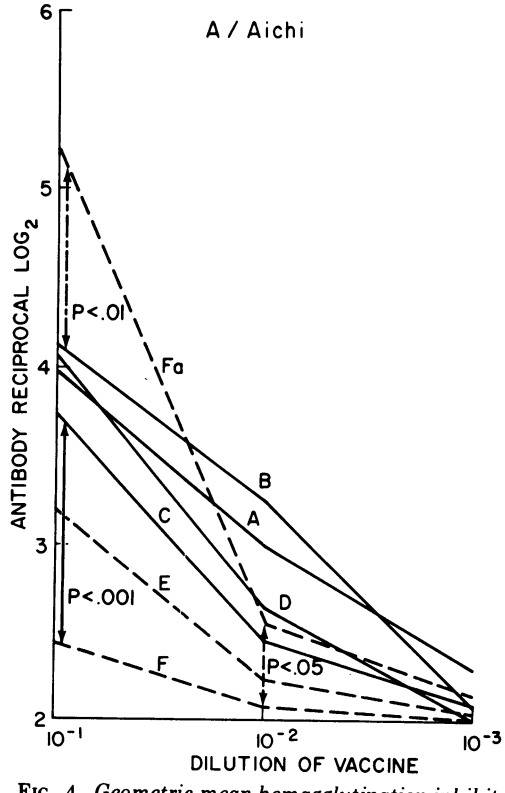


FIG. 4. *Geometric mean hemagglutination inhibiting antibody response to A/Aichi antigen at differing dilutions of vaccine. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines. Vaccine Fa is vaccine F adsorbed to $AlPO_4$.*

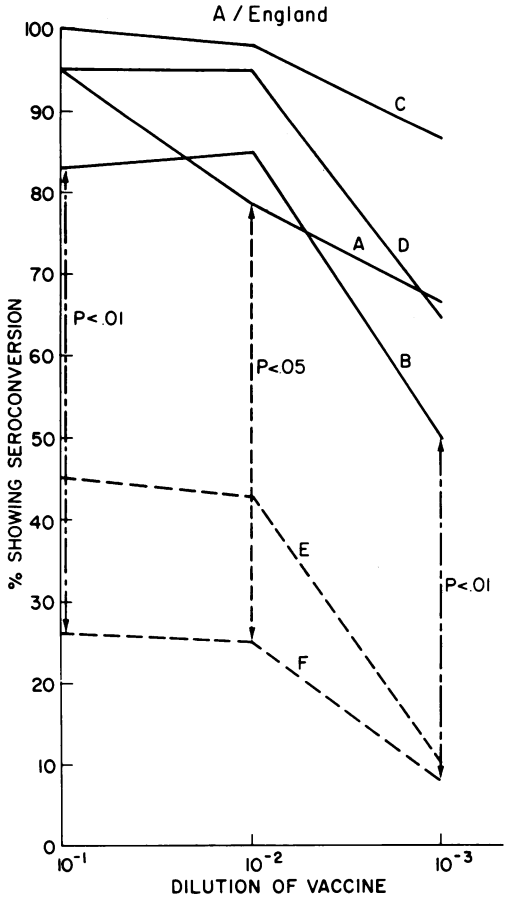


FIG. 5. *Homotypic seroconversion rates after inoculation with varying dilutions of bivalent vaccine containing A/England antigen. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines.*

CCA units of TBP-treated A/England and B/Mass vaccine and over 87 and 37 CCA units of ET vaccine were needed to produce the same conversion rate. Similarly, antibody could be induced in 50% of the mice by from 0.58 to 1.87 CCA units of whole-virus B/HK vaccine, whereas 24.5 and 18 CCA units were needed of TBP and ET vaccines, respectively. Such 50% minimal immunizing dose differences are less prominent when A/Aichi vaccines are considered, but the trend in the immunogenic inferiority of split-product vaccines is made evident by the fact that two to five times more CCA antigen of the split-product vaccines was required to produce 50% seroconversion when compared with the least immunogenic whole-virus vaccine.

## DISCUSSION

This study indicates the distinct immunogenic inferiority of split-product influenza vaccines of approximately equivalent CCA levels in mice. This was evident in a variety of both A and B vaccines tested and was particularly prominent and statistically significant for those vaccines treated with Tween-80 and ether. These findings are consonant with an earlier report by Davenport (5), who noted that experimentally produced vaccines made from A/PR/8, F/FM1/47, A/AA/2/60, B/Lee/40, and B/AA/3/62 viruses, which were treated with Tween-80 and ether and adsorbed to and eluted from $BaSO_4$, were less protective in mice than whole-virus vaccines containing somewhat more hemagglutination units and adsorbed to $AlPO_4$. He found, nevertheless, that adsorption of such

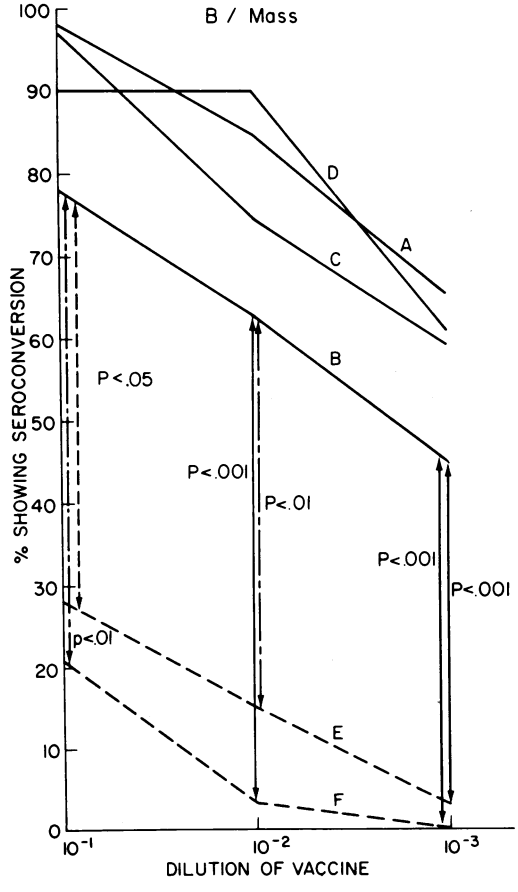


FIG. 6. *Homotypic seroconversion rates after inoculation with varying dilutions of bivalent vaccine containing B/Mass antigen. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines.*

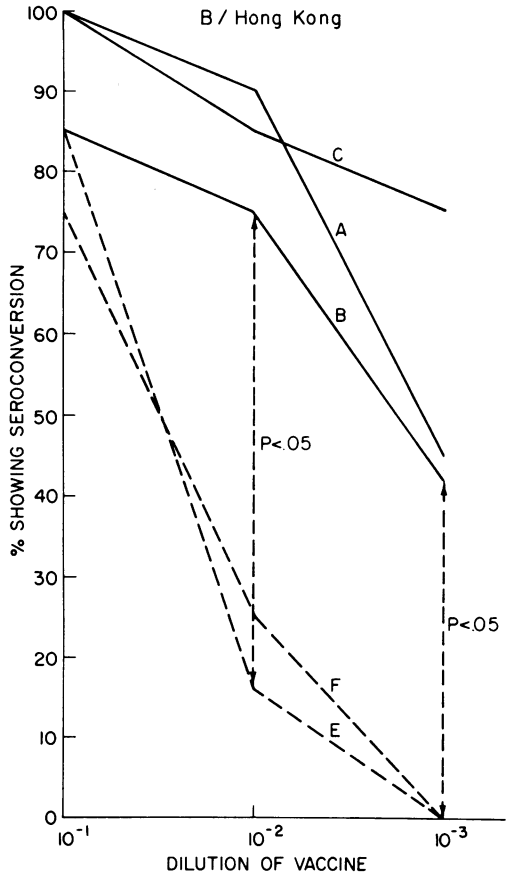


FIG. 7. *Homotypic seroconversion rates after inoculation with varying dilutions of monovalent B/Hong Kong vaccine. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines.*

a subunit vaccine to $AlPO_4$ markedly diminished such a difference. Neurath et al. (23) also noted that noncommercially produced ET and TBP vaccines made from A2/Japan and A2/Taiwan viruses appeared to be somewhat less immunogenic in mice than nondisrupted vaccine, although none of the vaccines were quantitated by the CCA test. Fenters et al. (12), however, noted that the immunogenicity in mice of a prototype Tween-80 and ether-treated split-product influenza vaccine made from an A2/Taiwan strain was superior to that produced by a whole-virus vaccine of equivalent antigenic content. The superiority noted was small and was seen only after a booster dose of vaccine had been given. As in the previous two studies mentioned (5, 23), the antigenic content of the split-product vaccines was not quantitated by the CCA test and the results were not subjected to statistical analysis. Our own studies indicate that when the antigenic content of modern, highly purified, commercially produced influenza vaccines is quantitated by the CCA test —currently the only official potency test of inactivated influenza vaccines in the U.S.—a much greater antigenic mass of split-product than of whole-virus vaccine is required to produce a given rate of seroconversion (i.e., 50%) in mice. Although none of the mice were challenged with a virulent mouse-adapted homologous strain of influenza virus, it could be expected that those receiving the whole-virus vaccines would be more protected from infection than those receiving the split-product vaccines, since the correlation between HI antibodies produced after parenteral vaccination of mice and protection from infection has been shown to be very close (10, 17). Such a correlation also exists in man (8, 16). The immuno-

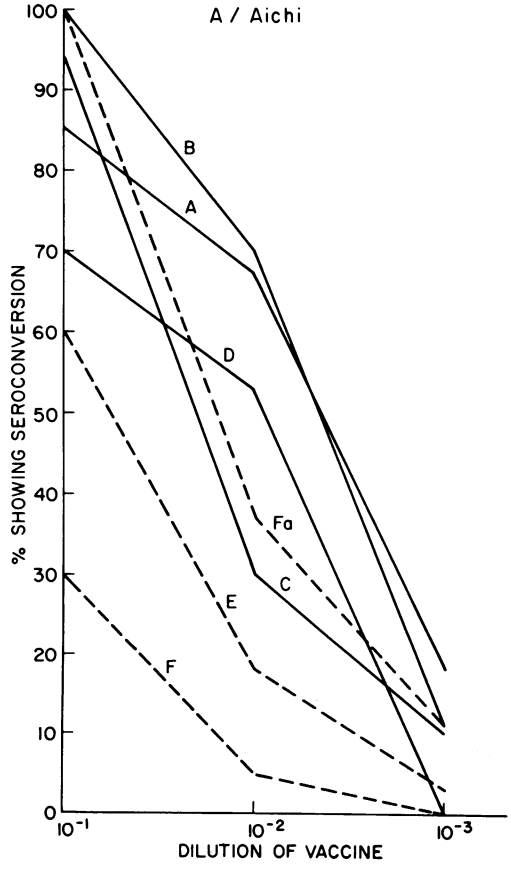


FIG. 8. *Homotypic seroconversion rates after inoculation with varying dilutions of monovalent A/Aichi vaccine. See Tables 1 and 2 for strain, type, and CCA content of lettered vaccines. Vaccine Fa is vaccine F adsorbed to $AlPO_4$.*

TABLE 3. *Fifty percent minimal immunizing dose ($MID_{50}$)*

| Manufacturer | $MID_{50}$ (in CCA units) | | | |
|---|---|---|---|---|
| | A/England | B/Mass | B/HK | A/Aichi |
| A | 0.85 | 0.31 | 0.86 | 4.9 |
| B | 1.68 | 1.50 | 1.87 | 3.2 |
| C | <1.0 | 0.67 | 0.58 | 14.9 |
| D | <0.73 | 0.39 | | 20.4 |
| E | >78 | >34 | 24.5 | 45 |
| F | >87 | >37 | 18 | >97 |
| Fa | | | | 10.4 |

genic inferiority of split-product vaccines in mice was evident with two type A and two type B influenza vaccines used in recent years, although it was less noticeable with vaccines made from A/Aichi prototypes than with those made from A/England, B/Mass, or B/Hong Kong prototypes. The slightly diminished immunogenicity of whole-virus A/Aichi vaccine might be related to its prolonged storage (over 2 years) before use or to previously described interstrain variations in the immunogenicity of influenza in animals (21, 22).

The cause of the diminished antibody response induced by split-product vaccines remains speculative. Possible differences in total antigenic mass, although clearly established as a major factor in the amount of serum antibody produced after influenza immunization in mice (9), does not seem to be a likely explanation, since simple adsorption of the ET vaccine to $AlPO_4$, a mineral carrier without intrinsic antigenicity, markedly improved the immunogenicity of the product equal to or better than that of whole-virus vaccines not adsorbed to such an adjuvant. It has been thought (5, 18) that the size of the split-product antigen might not be optimal for effective processing by the immune system and that adsorption to $AlPO_4$ might sufficiently increase the size so that antigenic expression would be maximal. Although type-specific ribonucleoprotein has been shown to be an important factor in the induction of HI antibodies after revaccination with another strain of the same type (29, 30), its significance in primary immunization remains to be studied further.

Whether the immunogenic inferiority of split-product vaccines noted in mice may exist in other species is somewhat difficult to determine. Rabbits demonstrate a poor serological response to influenza vaccines treated with deoxycholate or sodium dodecyl sulfate (11, 39) but show an antibody response to ET vaccines equivalent to that elicited by the whole-virus vaccines (39). Numerous studies (1-4, 6, 14, 15, 28, 32, 33) using ET or TBP split-product vaccines in humans have been performed which indicate that their efficacy in producing sufficient serum antibodies and protection from infection appears to be approximately equal to that of whole-virus vaccines. Precise comparison between the two types of vaccines is difficult in some reports, however, because of a variety of factors, including different routes of administration (32), the use of adjuvants (4, 14), or the use of tests other than the CCA test to quantitate the amount of antigen given (1, 3, 6, 14). An additional factor to consider when comparing results of studies with influenza vaccines in humans and in mice is the fact that the serological response in mice is of the primary type, whereas most previous studies comparing split-product and whole-virus influenza vaccines were performed in adults with some pre-existing experience with influenza and whose serological response would therefore be anamnestic in character. Whether subunit vaccines may be poor primary immunizing agents for seronegative individuals, who have a particular risk of influenza infection, but sufficient boosting antigens in man is equally difficult to discern, since previous studies in infants who presumably had little prior experience with influenza have shown that both ether-split (1, 15) and whole-virus (1) vaccines produced a poor serological response.

Since little is known about the precise relationship between mouse immunogenicity tests and protection from influenza infection in man (13), we are presently conducting a number of carefully controlled field studies in a variety of populations, including children, normal adults, and patients with chronic pulmonary disease, in an effort to determine whether the diminished immunogenicity of split-product vaccines in mice might also be noted in man. Results from these studies will also be correlated with other in vitro parameters of antigen quantitation including protein determinations, particle counts by electron microscopy, and polyacrylamide gel electrophoresis in order to find the most reproducible and reliable laboratory test of inactivated influenza vaccine potency.

## ACKNOWLEDGMENTS

We wish to thank Alfred Segee, Joan C. Enterline, Susan J. Lucas, and Donald J. McDorman for their excellent technical assistance, and F. A. Ennis for his critical comments and suggestions associated with a review of the manuscript.

## LITERATURE CITED

1. Brandon, F. B., C. D. Barrett, A. E. Hook, and G. O.

Lease. 1967. Human febrile response to influenza virus or its ether isolated hemagglutinins. Proc. Soc. Exp. Biol. Med. **125**:683-686.
2. Brandon, F. B., F. Cox, G. O. Lease, E. A. Timm, E. Quinn, and I. W. McLean. 1967. Respiratory virus vaccines. III. Some biological properties of Sephadex-purified ether extracted influenza virus antigens. J. Immunol. **98**:800-805.
3. Brandon, F. B., F. Cox, E. Quinn, E. A. Timm, and I. W. McLean. 1969. Influenza immunization—clinical studies with ether-split subunit vaccines. Bull. W.H.O. **41**:629-637.
4. Cromwell, H. A., F. B. Brandon, I. W. McLean, and J. F. Sadusk. 1969. Influenza immunization—a new vaccine. J. Amer. Med. Ass. **210**:1438-1442.
5. Davenport, F. M. 1968. Antigenic enhancement of ether-extracted influenza virus vaccines by $AlPO_4$. Proc. Soc. Exp. Biol. Med. **127**:587-590.
6. Davenport, F. M., A. V. Hennessy, F. M. Brandon, R. G. Webster, C. D. Barrett, and G. O. Lease. 1964. Comparisons of serologic and febrile response in humans to vaccination with influenza A viruses and their hemagglutinins. J. Lab. Clin. Med. **63**:5-13.
7. Eddy, B. E. 1947. A study of influenza virus vaccines by a serum virus neutralization test and by active immunization. J. Immunol. **57**:195-202.
8. Eickhoff, T. C. 1971. Committee on immunization. Immunization against influenza: rationale and recommendations. J. Infect. Dis. **123**:446-454.
9. Fazekas de St. Groth, S. F., and M. Donnelly. 1950. Studies in experimental immunology of influenza. III. The antibody response. Aust. J. Exp. Biol. Med. Sci. **28**:45-60.
10. Fazekas de St. Groth, S. F., and M. Donnelly. 1950. Studies in experimental immunology of influenza. IV. The protective value of active immunization. Aust. J. Exp. Biol. Med. Sci. **28**:61-75.
11. Fazekas de St. Groth, S. F., R. G. Webster, and F. M. Davenport. 1969. The antigenic subunits of influenza viruses. The homologous antibody response. J. Immunol. **103**:1099-1115.
12. Fenters, J. D., H. M. Yamashiroya, R. F. Petzold, and V. K. Tolkacz. 1970. Enhanced immunogenicity in mice of a purified, Tween-ether treated influenza vaccine. Appl. Microbiol. **20**:544-550.
13. Heller, L. 1967. The precision of three different types of response metameter used in mouse protection assays of the relative potencies of inactivated influenza virus vaccines. Int. Symp. Biol. Assay Methods Vacc. Sera **10**:33-42.
14. Hennessy, A. V., and F. M. Davenport. 1966. Relative antigenic potency in man of polyvalent influenza virus vaccines containing isolated hemagglutinins or intact virus. J. Immunol. **97**:235-238.
15. Hennessy, A. V., and F. M. Davenport. 1967. Vaccination of infants against influenza with polyvalent influenza hemagglutinins. J. Amer. Med. Ass. **200**:896-898.
16. Hobson, D., R. L. Curry, A. S. Beare, and A. Ward-Gardner. 1972. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J. Hyg. **70**:767-777.
17. Kaye, H. S., W. R. Dowdle, and J. L. McQueen. 1969. Studies on inactivated influenza vaccines. I. The effect of dosage on antibody response and protection against homotypic and heterotypic influenza virus challenge in mice. Amer. J. Epidemiol. **90**:162-169.
18. Maugh, T. H. 1973. Influenza. II. A persistent disease may yield to new vaccines. Science **180**:1159-1162.
19. McLean, I. W. 1961. Influenza virus vaccine production. Amer. Rev. Resp. Dis. **83**(Suppl.):157-159.
20. Miller, G. L., and W. M. Stanley. 1944. Quantitative aspects of the red blood cell agglutination test for influenza virus. J. Exp. Med. **79**:185-195.
21. Nakamura, K. 1965. Pathogenicity and immunogenicity of various strains of influenza virus for mice. Biken J. **8**:155-165.
22. Neurath, A. R., and B. A. Rubin. 1971. Viral structural components as immunogens of prophylactic value, p. 27-67. *In* J. L. Melnick (ed.), Monographs in virology, vol. 4. S. Karger, Basel.
23. Neurath, A. R., J. T. Stasny, B. A. Rubin, A. K. Fontes, W. A. Pierzchala, F. P. Wiener, and R. W. Hartzell. 1970. The effect of nonaqueous solvents on the quaternary structure of viruses: properties of hemagglutinins obtained by disruption of influenza viruses with tri (n-butyl) phosphate. Microbios **2**:209-224.
24. Norrby, E., R. Lagercrantz, and S. Gard. 1965. Measles vaccination. III. Serological responses to immunization with purified hemagglutinin. Acta Paediat. Scand. **54**:581-586.
25. Norrby, E., R. Lagercrantz, and S. Gard. 1966. Measles vaccination. V. The booster effect of purified hemagglutinin in children previously immunized with this product or formalin-killed vaccine. Acta Paediat. Scand. **55**:73-78.
26. Perkins, F. T. 1969. Control of influenza vaccine, with special reference to experience in the United Kingdom. Bull. W.H.O. **41**:554-555.
27. Perkins, F. T. 1973. Report of an informal meeting of manufacturers and the Control Laboratory on influenza vaccine. J. Biol. Stand. **1**:195-197.
28. Phillips, C. F., C. A. Phillips, W. E. Hodgkin, B. R. Forsyth, B. A. Rubin, and M. E. Geraghty. 1973. Killed subunit influenza vaccine in children. Pediatrics **52**:416-419.
29. Potter, C. W., R. Jennings, C. McLaren, and R. C. Rees. 1973. Antibody response of hamsters to $A_2$/Hong Kong virus vaccine after priming by heterotypic virus infection. Infect. Immunity **8**:137-144.
30. Potter, C. W., R. Jennings, W. M. Marine, and C. McLaren. 1973. Potentiation of the antibody response to inactivated $A_2$/Hong Kong vaccines by previous heterotypic influenza virus infection. Microbios **8**:101-110.
31. Ruben, B. A., W. A. Pierzchala, and A. R. Neurath. 1967. Elicitation of antibody response against influenza viruses by different viral subunit preparations. Arch. Gesamte Virusforsch. **20**:268-271.
32. Ruben, F. L., L. W. Akers, E. B. Stanley, and G. G. Jackson. 1973. Protection with split and whole virus vaccines against influenza. Arch. Intern. Med. **132**:568-571.
33. Ruben, F. L., and G. G. Jackson. 1972. A new subunit influenza vaccine: acceptability compared with standard vaccines and effect of dose on antigenicity. J. Infect. Dis. **125**:656-664.
34. Ruben, R. J., and M. B. Gregg. 1973. English flu—a primer. N. Engl. J. Med. **288**:467-468.
35. Schulman, J. L., and E. D. Kilbourne. 1971. Correlated studies of a recombinant influenza virus vaccine. II. Definition of antigenicity in experimental animals. J. Infect. Dis. **124**:463-472.
36. Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. **88**:320-329.
37. Tauraso, N. M., T. C. O'Brian, and E. B. Seligmann. 1969. Problems of influenza vaccine standardization. Bull. W.H.O. **41**:497-506.
38. Tauraso, N. M., F. A. Pedreira, S. L. Spector, and G. M. Bernier. 1971. Effect of various methods of removing non-specific inhibitors to virus hemagglutination upon serum proteins and immunoglobulins. Arch. Gesamte Virusforsch. **34**:214-222.
39. Webster, R. G., and W. G. Laver. 1966. Influenza virus subunit vaccines: immunogenicity and lack of toxicity for rabbits of ether and detergent-disrupted virus. J. Immunol. **96**:596-605.