Relative Merits of Aqueous and Adjuvant Influenza Vaccines *when used in a* Two-Dose Schedule

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A RECOMMENDATION that persons of all ages be vaccinated against influenza by giving them two doses of vaccine was first made in 1957 by the Commission on Influenza, Armed Forces Epidemiological Board. This recommendation stemmed from the demonstration that the primary antibody response of man to influenza virus vaccines seemed inadequate. For example, infants and children respond irregularly and poorly to a single dose of influenza virus vaccine (*1–5*). Young adults and persons over 30 likewise fail to develop uniformly high antibody levels when vaccinated once with those strains of influenza virus which they have not previously encountered by infection (*2,6–8*).

Fortunately, it had been clearly established that vaccination was capable of laying the foundation for a "booster" response in children and adults (*3,4*). Hence, in order to meet the emergency when the antigenically novel Asian strains appeared in 1957, the recommendation of a two-dose schedule of vaccination could be made with confidence.

Since 1957 a schedule of two doses of influenza virus vaccine for protection against influenza has been adopted by the military and recommended to segments of the civilian population (*9,10*). Meanwhile, general interest has accumulated in the use of those adjuvant influenza virus vaccines emulsified in mineral oil that have been under development since 1951 by the Commission on Influenza. Consequently, it seems timely to report on the results of a series of investigations undertaken to compare antibody responses of humans given either two doses of aqueous vaccine, two doses of adjuvant vaccine, or one dose of vaccine of one kind followed by a second dose of the other kind.

The findings indicate that mineral oil adjuvant vaccine, when used as one or both inoculations in a two-dose schedule, is remarkably effective for stimulating high, broad, uniform, and persistent antibody levels against prototype strains of influenza A viruses, regardless of prior natural exposures. Furthermore, when two doses of adjuvant vaccine are given within an interval of 2 to 3 months, a

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phenomenal economy can be effected in the requirement of antigen.

Materials and Methods

The vaccines used in the study were prepared through the courtesy of commercial biologics firms. Concentration and purification of virus were accomplished by centrifugation. Virus suspensions were inactivated with formalin (1:4,000), and merthiolate (1:10,000) was added as a preservative. Adjuvant vaccines were made by emulsifying one volume of virus concentrate with an equal volume of a mixture containing nine parts light medicinal mineral oil (Drakeol 6–VR, Pennsylvania Refining Co.) and one part emulsifier (purified Arlacel A, Atlas Powder Co.). These adjuvant vaccines are experimental products and are not commercially available.

A volume of 0.25 ml. of adjuvant vaccine was given intramuscularly in the posterior belly of the triceps muscle. Aqueous vaccine was administered subcutaneously in 1.0 ml. doses.

Except in one instance, antibody response to vaccines was studied in young children aged 4 to 11 years (median, 8 years) and in adults 30 or more years of age (median, 45 years). The exception was a series of experiments conducted between December 1956 and April 1957 on four groups of children 11 to 16 years of age to determine the minimal amount of virus necessary for a satisfactory booster response after an initial dose of adjuvant preparation.

The serum was promptly separated from each blood sample and merthiolate (1:1,000) was added to yield a final concentration of 1:10,000. Serums were stored at 4° C. and heated to 56° C. for 30 minutes prior to use. For measurement of antibody against Asian virus, serums were treated with two parts M/90 KIO_4 for 12 to 18 hours at 4° C. Excess periodate was then neutralized by addition of five parts of a 10 percent solution of glycerol in saline.

Hemagglutination inhibition activities of serums were determined by a pattern test with four units of virus and 0.5 percent chicken erythrocytes suspended in saline (*11*). Antibody titers are expressed as reciprocals of initial dilutions of serums.

Strains of influenza virus used for vaccination and for antibody determination were chosen from the virus collection of this laboratory. They were: swine 1976 (1931), PR8 (A–1934), PR301 (A′–1954), AA/1 (A′–1957), and AA/23 (Asian–1957).

Saline refers to 0.15 M sodium chloride buffered at pH 7.2 with 0.01 M phosphate.

Results

Secondary antibody response of children to adjuvant or aqueous vaccines after initial vaccination with either product. In order to ascertain the sequence of vaccines that yields optimal antibody responses in children, groups of 25 children were given either adjuvant or aqueous polyvalent influenza virus vaccine in May 1957 and 7½ months later, in December 1957, were injected with the same vaccine or with vaccine of the opposite kind. The adjuvant vaccine contained 60 chicken cell agglutination (CCA) units each of swine (A), PR8 (A), and PR301 (A′) strains per dose. The aqueous vaccine contained, per dose, 200 CCA units of each of the same viruses.

Table 1 shows antibody responses measured by hemagglutination inhibition in serums obtained 2 weeks after revaccination with aqueous

Table 1. Antibody response of children to a second dose of aqueous or adjuvant vaccine after an initial dose of either vaccine

Polyvalent vaccine	Test strain and antibody response [1]		
	Swine	PR8	PR301
Aqueous; [2] aqueous: [3]			
Before second dose	30	29	102
After second dose	179	512	307
Aqueous; [2] adjuvant: [3]			
Before second dose	42	21	83
After second dose	922	2,458	1,226
Adjuvant; [2] aqueous: [3]			
Before second dose	154	205	768
After second dose	410	870	1,331
Adjuvant; [2] adjuvant: [3]			
Before second dose	242	141	768
After second dose	1,229	1,741	1,818
Controls:			
May 1957 [4]	8	8	16
December 1957 [5]	8	8	24

[1] Geometric mean antibody titer. [2] Initial dose. [3] Second dose. [4] Before experiment. [5] After experiment.

vaccine and 6 weeks after revaccination with adjuvant vaccine. Antibody levels found in serums of a control group of nonvaccinated children from whom blood specimens were taken before and at the conclusion of the experiment are included. Note that, as previously reported, in children the response and persistence of antibody following the first inoculation of either aqueous or adjuvant vaccine was markedly higher against the A′ strain than against swine or PR8 virus. The reason for this discrepancy in response to these antigenically representative strains given by vaccination is that these children were born after the period of prevalence of the latter antigenic types of influenza A strains (*12,13*), and had acquired familiarity with A′ antigens by natural infection but were inexperienced or unfamiliar with swine and PR8-like antigens.

However, two doses of polyvalent vaccine of either the aqueous or adjuvant type overcame the limiting effect of their deficiencies in antigenic experience. Two doses of aqueous vaccine induced higher and broader antibody levels against the prototype strains used for vaccination and testing than did a single dose of aqueous vaccine. The antibody response after the second dose of aqueous vaccine was not too different from that found after a single dose of adjuvant vaccine. More importantly, aqueous vaccine followed by adjuvant vaccine, and adjuvant vaccine followed by aqueous vaccine, resulted in higher and broader antibody levels than were induced by two doses of aqueous vaccine. In this age group, the highest and most uniform antibody levels were achieved by two doses of adjuvant vaccine. The nonvaccinated children showed no significant antibody increase to these strains during the period of study, even though an epidemic of Asian influenza occurred among them in October.

These data, taken with other examples, reemphasize the conclusion that influenza virus vaccines emulsified with mineral oil have distinct advantages for immunization of man (*14–19*). To explore the problem further, concomitant experiments of identical design were carried out in adults.

Secondary antibody response of adults to adjuvant or aqueous vaccines after initial vaccination with either preparation. Four groups of 25 adults each, all over the age of 30 years, were vaccinated and revaccinated with the same vaccines and with the same dosages and schedules as were used for children. The antibody responses of these adults, measured by hemagglutination inhibition, are shown in table 2.

Table 2. Antibody response of adults over 30 years of age to a second dose of aqueous or adjuvant vaccine after an initial dose of either vaccine

Polyvalent vaccine	Test strain and antibody response [1]		
	Swine	PR8	PR301
Aqueous; [2] aqueous: [3]			
Before second dose	230	166	24
After second dose	333	333	45
Aqueous; [2] adjuvant: [3]			
Before second dose	192	128	51
After second dose	1, 229	922	358
Adjuvant; [2] aqueous: [3]			
Before second dose	1, 843	1, 331	512
After second dose	2, 867	2, 458	768
Adjuvant; [2] adjuvant: [3]			
Before second dose	1, 843	1, 024	461
After second dose	3, 686	2, 048	1, 024
Controls:			
May 1957 [4]	58	14	10
December 1957 [5]	109	21	13

[1] Geometric mean antibody titer. [2] Initial dose. [3] Second dose. [4] Before experiment. [5] After experiment.

Again, the effect of prior conditioning by natural infections upon antibody response to influenza virus vaccines is seen in this age group. The difference in the response of children and adults is explainable by the fact that these adults had their primary exposure to swinelike viruses in their childhood, and later in their lives were exposed to A and A′ strains (*12–13*).

In consequence, the antibody response to the first dose of either kind of vaccine is greatest to swine strain, next to PR8, and least to the A′ test virus. Unlike the result in children, two doses of aqueous vaccine did not appear to cause an important increase in the height or breadth of antibody response to the prototype strains given. Apparently persons of this age group are refractory to successive stimuli with aqueous influenza virus vaccines of this potency.

In contrast, a single dose of adjuvant vaccine induced higher and broader antibody levels in

adults than did two doses of aqueous vaccine. In addition, the results after alternate vaccination with two kinds of vaccine are somewhat different from the findings in children. Polyvalent adjuvant vaccine, followed by polyvalent aqueous vaccine, yielded higher and more uniform antibody levels against all test strains than did the reverse combination. Apparently adjuvant vaccine overcomes the refractory state shown by the failure of persons over 30 years of age to respond very actively to successive doses of aqueous vaccine alone.

The first dose of aqueous vaccine does not appear to condition the adult cohort sufficiently for them to respond optimally when given the later dose of adjuvant vaccine. Conversely, the first dose of adjuvant vaccine appears to lay a more effective foundation for the second stimulus since even though it was given as an aqueous preparation, further enhancement of all antibody levels was thereby achieved. On the average, as in children, the best result in adults came from two doses of adjuvant vaccine. Antibody increase in the unvaccinated group was minimal and apparently was caused by infection with Asian strains since an outbreak of Asian influenza was identified in this area during the investigation.

The results of the experiments indicate that, in both children and adults, with current products, optimally broad and high antibody levels against the prototype strains of influenza A viruses can best be induced by vaccination with two doses of adjuvant vaccine. Less advantageous, though clearly effective, is a schedule that alternates successively the use of aqueous and adjuvant vaccines. As has been shown, the final antibody yield under these circumstances is considerably influenced by age and its concomitant status of prior antigenic exposure (*3,4*). Least advantageous is a schedule of two doses of aqueous vaccine.

Persistence of antibody after two doses of polyvalent influenza virus vaccine. One of the advantages of adjuvant vaccine is the persistence of antibody at high levels for a long period. To compare the persistence of antibody after vaccination with aqueous or adjuvant vaccines given in the two-dose combinations used, blood specimens were taken from both children and adults 1 year following the second vaccination, and antibody levels were determined. Table 3 shows the geometric mean antibody titers observed after the second vaccination and 1 year later.

In children, antibody titers induced by two doses of polyvalent aqueous vaccine had fallen to low levels by the end of 1 year, as had the levels achieved with polyvalent adjuvant vaccine followed by polyvalent aqueous vaccine. Although the levels produced by two doses of adjuvant vaccine, or by aqueous vaccine followed by adjuvant vaccine, fell in the year's interval, high levels against the vaccine strains were still present.

In adults too, residual antibody levels were

Table 3. Geometric mean antibody titers of three test viruses in children and in adults 6 weeks and 1 year after a second inoculation with aqueous or adjuvant vaccine

Polyvalent vaccine	Time after vaccination	Geometric mean antibody titer					
		Children (4–11 years)			Adults (over 30 years)		
		Swine	PR8	PR301	Swine	PR8	PR301
Aqueous	6 weeks	216	410	563	131	192	77
Aqueous	1 year	18	24	102	83	83	29
Aqueous	6 weeks	563	2, 662	2, 662	1, 024	512	307
Adjuvant	1 year	96	512	1, 126	614	307	205
Adjuvant	6 weeks	256	1, 024	3, 891	2, 613	1, 131	973
Aqueous	1 year	19	32	614	563	205	256
Adjuvant	6 weeks	1, 131	819	1, 046	1, 536	922	1, 843
Adjuvant	1 year	307	166	666	666	291	486

lowest in those persons who received two doses of aqueous vaccine. In contrast, antibody levels found 1 year after the second inoculation of vaccine were high against all strains in serums of the three groups whose schedules included adjuvant vaccine.

The findings demonstrate that in children persistence of high and broad antibody levels for 1 year required the use of adjuvant vaccine for the second dose. In adults, antibody persistence was not dependent, apparently, upon whether the adjuvant vaccine was given first, last, or twice.

Relation of virus content in primary adjuvant vaccine to conditioning for secondary response. Mineral oil adjuvant vaccines have been shown to be useful not only because they provoke high antibody levels that persist, but, in addition, considerably less antigen is required when the antigen is incorporated in a water-in-oil emulsion (*14,19*). To date the studies on conservation of influenza antigens by the use of mineral oil adjuvants have been limited to the results following a single dose of vaccine. It was of interest, therefore, to ascertain the minimal amount of virus that was capable of setting the stage for a satisfactory booster response when the initial dose was given as an adjuvant preparation. For this purpose, four groups of 25 children each, ages 11 to 16 years, were given an adjuvant vaccine containing either 1, 10, 50, or 250 CCA units of swine strain. Five months later, blood specimens were taken from the subjects, and they were revaccinated with an aqueous vaccine containing 200 CCA units each per dose of swine, PR8, and PR301 strains. Final serum samples were obtained 2 weeks later.

The experiments were conducted between December 1956 and April 1957. A small outbreak of influenza A′ occurred in this region during that period. Swine strain was chosen for the first vaccine stimulus, because the children had not been previously exposed to that virus, and their serums were generally devoid of antibodies to it (*12,13*). For the secondary vaccine stimulus, a polyvalent vaccine containing swine strain was employed, because polyvalent vaccines are in general use for protection against influenza.

The results of antibody determinations in serum samples obtained from each group before and after the booster dose of aqueous vaccine are summarized in table 4. For comparison, antibody levels found in a fifth group of 25 children given a single dose of the same aqueous vaccine are shown.

Apparently, no conditioning of the antibody-forming mechanisms had occurred with the first vaccination in the group given 1 CCA unit of swine adjuvant vaccine. When individuals in the group who had received 10 CCA units of swine virus were revaccinated, the antibody response was markedly greater, indicating that an important conditioning had occurred follow-

Table 4. Effect of varying amounts of swine virus in adjuvant vaccine given as a primary stimulus upon antibody response to a "booster" vaccination with aqueous vaccine

Primary vaccination [1] (CCA units swine strain)	Serum examined	Geometric mean antibody titer
1	5 months after first dose [2]	8
	2 weeks after second dose	19
10	5 months after first dose [2]	22
	2 weeks after second dose	154
50	5 months after first dose [2]	102
	2 weeks after second dose	230
250	5 months after first dose [2]	128
	2 weeks after second dose	410
Control: [3]		
Prevaccination serum		8
Postvaccination serum		18

[1] Adjuvant vaccine.
[2] Revaccinated with aqueous vaccine containing 200 CCA units swine, PR8, and PR301 strains per dose.
[3] Single dose aqueous vaccine.

ing that first stimulus. In fact, by increasing the amount of virus used for the primary dose fivefold (50 CCA units) or 25-fold (250 CCA units) the yield of antibody after the booster dose was only 1.5 times and 2.7 times higher, respectively, than was obtained with but 10 CCA units. However, the "prebooster" titer was much higher after the 50 and 250 CCA unit initial dose.

These results suggested that the most economic "cutoff point" for selecting a dose for primary immunization with an adjuvant vaccine might be 10 CCA units, and clearly indicated that relatively small amounts of virus are capable of laying the foundation for a secondary response if given in the emulsified form.

Antibody response following two small doses of polyvalent adjuvant vaccine. The success obtained in minimizing the amount of virus needed for primary stimulation by vaccination, as described in the preceding section, prompted studies designed to determine whether comparably small amounts of virus could be used per strain in a polyvalent vaccine to be given for both primary and "booster" inoculations. At the same time, the question of shortening the interval between injections was investigated.

To these ends, two groups of 25 children each were inoculated with a polyvalent adjuvant vaccine containing 10 CCA units of swine, PR8, A′, and Asian strains (40 CCA unit vaccine). Eight weeks after the primary vaccination blood specimens were taken from the first group and they were revaccinated; 12 weeks after the first injection blood specimens were taken from the second group and they were revaccinated. For comparison, a third group received a more potent vaccine containing 50 CCA units each per dose of swine, PR8, and A′ virus strain and 100 CCA units of Asian virus (250 CCA unit vaccine); blood specimens were taken from them and they were revaccinated with the same preparation 12 weeks later. Final serum specimens were obtained from each group 8 weeks after the second vaccination. These investigations were carried out from December 1958 to February 1959. Influenza was not observed in this area during that interval. Table 5 shows the geometric mean antibody titers found for these groups before and after the second vaccination.

As observed in previous experiments of this series, in children antibody response to the first vaccination was poor as measured against swine and PR8 strains, but good against A′ virus. Moreover, this effect was, for practical purposes, independent of the strength of vaccine given. Note that the response as measured by Asian virus was excellent, indicating that these subjects has been already conditioned by infection as early as the summer of 1958, when the experiments were conducted, to respond well to a single vaccination with preparations of either potency.

A second inoculation of the small dose of adjuvant vaccine 8 weeks after the first dose resulted in reinforcement of antibody to all antigens present in the vaccine. When the interval between inoculations was increased to 12 weeks, a greater reinforcement of antibody occurred against swine, PR8, and Asian strains.

Table 5. Antibody response in children after two doses of adjuvant vaccine containing very small amounts of virus

Primary vaccination (CCA units adjuvant vaccine)	Revaccination	Geometric mean antibody titer			
		Swine	PR8	A′	Asian
40 (small dose)[1]	8 weeks after first dose	19	19	166	256
	8 weeks after second dose	102	96	512	717
40 (small dose)[1]	12 weeks after first dose	22	22	179	358
	8 weeks after second dose	230	230	512	922
250 (standard dose)[2]	12 weeks after first dose	24	18	166	512
	8 weeks after second dose	256	90	461	1, 229

[1] 10 units each swine, PR8, A′, and Asian strains.
[2] 50 units each swine, PR8, A′; 100 units Asian strain.

The 250 CCA unit vaccine given twice at a 12-week interval gave antibody levels not significantly different from those obtained after two doses of 40 CCA unit vaccine given 3 months apart.

These results would appear to establish the principle that an enormous saving in antigens can be effected when very small amounts of influenza viruses are incorporated in emulsions made with mineral oil and used at appropriate intervals for primary and booster inoculations. Application of this method to other virus-antibody systems would seem appropriate.

Discussion

The results of the present study appear to be especially pertinent to the problem of protection against influenza by vaccination, and, in addition, may have general implications for the use of killed-virus vaccines.

As demonstrated in the present study, satisfactory high, broad, and durable antibody levels against influenza A viruses cannot be obtained currently, except by the use of adjuvant vaccine employed as part of a two-dose schedule of inoculations. The adjuvant vaccine may be used for both doses, or for the second dose. The high antibody levels obtained should provide a larger margin of security for protection, since they appear to be well above the minimal level needed for resistance to infection (*20*). The breadth of antibody response induced is deemed advisable because such a composite antibody appears to provide a firmer basis for resistance, which is not as readily overcome by antigenic variation in strains that may prevail after a vaccine has been given.

The persistence of antibody at high levels after vaccination bears promise of eliminating the necessity for annual revaccination against influenza. The exact period of time that may elapse before a third dose of adjuvant vaccine is required is, as yet, undetermined, but in this connection it is pertinent to point out that persistence for 3 years of high levels of antibody following a single dose of adjuvant vaccine has been observed by Davis and associates (*21*). Elimination of the necessity for annual revaccination should promote acceptance of influenza virus vaccines by physicians and the general public.

The demonstration that two small doses of virus in adjuvant can induce high antibody levels in a short time even to strains of influenza viruses with which the subjects had had no prior antigenic experience, is an important finding. There are several practical implications of this observation.

Obviously, it would have been an advantage in the summer of 1957 to have been able to immunize persons effectively against Asian influenza, using only a total of 20 CCA units of an Asian strain given in divided doses 8 weeks apart. Since the minimum potency of aqueous vaccine used in that emergency contained 200 CCA units of Asian virus, the procedure outlined would have effected a tenfold saving in antigen and at the same time probably would have yielded higher titers of antibody than those resulting from vaccination with the aqueous product. Stated another way, the limited stocks of Asian antigen available that summer could have been utilized to offer protection to 10 times as many persons, with greater confidence in the degree of protection obtained. Consequently, the time needed for effective mobilization against the pandemic could have been shortened. In the face of the next pandemic of influenza, such a practice may prove helpful.

The use of two small doses of virus in adjuvant may also prove to be advantageous for vaccines that contain antigens which are costly to produce or which at best can only be grown at low titer. Conservation of antigen using types 4 and 7 of adenoviruses in an adjuvant vaccine has already been shown by Meiklejohn (*22*). Excellent protection against acute respiratory disease was demonstrated in recruits using but one-half the amount of adenovirus licensed for incorporation in an aqueous product. Moreover, the same vaccine contained 50 CCA units each of six strains of influenza viruses, and was equally effective in protecting against Asian influenza (*22*).

Clearly, the way seems open for the further development of multivirus vaccines containing in the same package different disease agents. At the speed with which new viruses are currently being isolated and identified (*23*), it is obviously desirable to have available a procedure for inducing protective antibodies

against the largest number of viruses at the lowest cost for antigen and with the least number of injections. In addition, it is desirable to have such antibodies persist for prolonged periods of time. At present, the most promising approach toward these goals is the use of small doses of virus in adjuvant given for primary and booster stimulation.

Summary

The findings of this study demonstrate that broad and high levels of antibody against all known families of influenza A virus may be induced in children and adults by either two doses of adjuvant polyvalent vaccine or by aqueous polyvalent vaccine followed by adjuvant polyvalent vaccine. Antibodies so produced remain at high levels for a long period of time while antibodies produced by two doses of aqueous vaccine fall to low levels in one year.

In children it was observed that two very small doses of virus in adjuvant vaccine gave high levels of antibody even when the interval between inoculations was only 8 weeks.

The findings reported indicate that high, broad, and persistent antibody levels can be achieved in man with a minimum of inoculations and materials. The implications of the results are discussed.

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