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A new surface-antigen-adsorbed influenza virus vaccine

II. Studies in a volunteer group

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SUMMARY

A group of 23 volunteers were each inoculated with 600 CCA of a new form of influenza virus A/England/42/72 vaccine; this vaccine consisted of purified haemagglutinin and neuraminidase antigens adsorbed to alhydrogel. No significant reactions to the vaccine were reported. Twenty-two volunteers produced increased titres of serum HI antibody, and all showed increased titres of NI antibody after immunization. Thus, for volunteers with no pre-immunization serum HI antibody, the geometric mean titre of serum antibody increased from 1/5 to 1/196 after immunization. Ten volunteers developed local neutralizing antibody after immunization; this antibody response was detected most frequently in volunteers who showed the greater serum antibody response to immunization, and in nasal washings with the higher concentrations of protein and IgA. Ten weeks after immunization, the vaccinees and a group of matched controls were inoculated intranasally with attenuated A/England/42/72 virus. Evidence of infection with the challenge virus was found in 14 of the control subjects and in one of the vaccinees. The results indicate that the surface-antigen-adsorbed vaccine induced high titres of serum antibody, and gave significant protection against challenge infection.

INTRODUCTION

Immunization with inactivated influenza virus, particularly immunization of young children, has been reported to cause frequent local and occasional systemic side reactions (Quilligan, Francis & Minuse, 1949; Davenport *et al.* 1964); however, these reactions were less common after immunization with subunit or split influenza virus vaccines. Thus, an influenza vaccine of either virus haemagglutinin or deoxycholate (DOC)-split virus produced less reactions than whole virus vaccine (Webster & Laver, 1966; Brandon, Barrett, Hooke & Lease, 1967). On the other hand, the results of immunization studies with subunit vaccines have suggested that these may be less immunogenic than whole virus vaccine. A purified haemagglutinin vaccine was reported to produce antibody titres comparable with

those produced by whole virus (Davenport *et al.* 1964; Hennessey & Davenport, 1966; Brandon *et al.* 1967), but a DOC-split virus vaccine and a haemagglutinin vaccine prepared by ether treatment were both found to be less antigenic (Webster & Laver, 1966; Davenport, 1968).

The present study reports the results of immunization with a vaccine containing purified haemagglutinin and neuraminidase antigens adsorbed to alhydrogel; it was hoped that this material would be less toxic than whole virus, and that adsorption to a carrier would potentiate the immunogenicity of the purified virus subunits (Davenport, 1968). A group of volunteers was immunized with the surface-antigen-adsorbed vaccine, and the serum antibody response to virus haemagglutinin and neuraminidase was determined. Four weeks after immunization, the vaccinees, together with a group of matched controls, were challenged with a homologous, attenuated virus given intranasally. The incidence of virus infection in the two groups was determined by virus isolation and serum antibody response.

MATERIALS AND METHODS

Study group

Fifty-two medical students volunteered for the study; all were healthy, with no history of allergy to eggs or egg products. Seventeen of the volunteers were female, and the ages ranged from 20 to 26 years.

Virus and virus vaccine

The MRC-7 virus (H3N2), a recombinant of influenza virus A/England/42/72 (H3N2) and A/PR/8/34 (H0N1), was kindly supplied by Dr A. S. Beare, Common Cold Research Centre, Harvard Hospital, Salisbury; the virus was supplied in allantoic fluid, and was stored at $-80°$ C. Immediately before use, the virus was thawed and diluted in phosphate buffered saline, pH 7·4, to contain $10^{7·5}$ EID 50/ml. For virus inoculation, the volunteers lay on an examination couch with the head fully extended backwards over the end of the couch, and 0·5 ml. of virus was inoculated dropwise into each nostril. After inoculation, the volunteers remained lying for 2 min., and on rising were asked not to blow their noses for a further hour.

The A/England/42/72 vaccine was supplied by Dr I. Furminger, Evans Biologicals Ltd, Speke, Liverpool. Briefly, the virus was grown in embryonated eggs and purified by zonal centrifugation. The concentrated virus was then treated with Triton N101 and the haemagglutinin and neuraminidase antigens separated from the other viral proteins, and adsorbed to an alhydrogel carrier (Brady & Furminger, in preparation). The vaccine was standardized to contain 600 international units (i.u./ml.).

Experimental design

A serum sample was taken from each volunteer and tested for haemagglutination-inhibiting (HI) antibody to influenza virus A/England/42/72. From these results, the volunteers were divided into two matched groups; each volunteer in the group to be immunized had a corresponding control whose serum HI antibody titre to A/England/42/72 virus was identical or very similar. If a volunteer dropped

out of the study, his corresponding number in the other group was also excluded. At the end of the study, each group contained 23 volunteers who had completed the programme.

Each volunteer in the test group was inoculated intramuscularly with 600 i.u. of the A/England/42/72 surface-antigen-adsorbed vaccine in a 1·0 ml. volume. Two nasal wash specimens were collected before immunization, and further specimens were collected weekly for 3 weeks after immunization; these specimens were collected and processed as described by Downie & Stuart-Harris (1970). Three weeks after immunization, a second blood specimen was taken, and seven days later the volunteers, together with the control group, were each inoculated intranasally with $10^{7\cdot5}$ EID50 of MRC-7 virus. Throat swabs, taken in medium '199' containing 2·0% bovine serum albumin and antibiotics, and nasal washings for virus isolation were collected 3 days after virus inoculation and a further blood sample was taken 18 days later.

Virus isolation

Nasal washings and throat swabs for virus isolation were stored at −80° C before testing. The specimens were thawed and each specimen was inoculated by the allantoic route into 10-day embryonated eggs. After 3 days incubation at 33° C., the allantoic fluids were harvested and tested for haemagglutinin with fowl cells. All viruses isolated were identified by HI tests using monospecific ferret antisera.

Serological tests

Haemagglutination-inhibition (HI) tests. These tests were carried out by a modification of the microtitre method (Sever, 1962). Before testing, serum specimens were treated with cholera filtrate (Burroughs Wellcome Ltd) for 18 hr. at 37° C., and subsequently heated for 30 min. at 56° C. Serum dilutions in phosphate buffered saline (PBS) were mixed with an equal volume of buffer containing eight (50%) haemagglutinating units of A/England/42/72 virus. After 10–15 minutes, a further volume of 0·5% fowl erythrocytes in PBS was added, and the cells allowed to settle at room temperature. The HI titres of the sera were expressed as the highest serum dilution which caused 50% reduction of virus haemagglutination.

Neutralization tests. All nasal washings were tested for neutralizing antibody to A/England/42/72 by the allantois-on-shell (AOS) method (Fazekas de St Groth, Witchell & Lafferty, 1958). Influenza virus A/England/42/72, at a concentration of $10^{3\cdot0}$ EID50 in a 0·05 ml. volume, was added to 0·5 ml. of serial dilutions of unconcentrated nasal washings. After incubation for 30 min. at 0° C., 0·05 ml. of each virus-nasal wash mixture was added to AOS fragments prepared from 10-day embryonated eggs. Shell fragments from four different eggs were used to test each dilution of nasal washing, since eggs vary in susceptibility to virus infection. After incubation for 72 hr. at 33° C. with constant shaking, the shell fragments were removed and the culture fluids tested for virus by haemagglutination. The neutralizing antibody titres were calculated by the method of Reed & Muench (1938).

Complement-fixation tests. These tests were carried out by the method of

Bradstreet & Taylor (1962), using 2·5 MHD50 of guinea-pig complement and A/England/42/72 virus soluble antigen extracted from the chorio-allantoic membranes of virus-infected eggs. The tests were incubated overnight at 4° C. before adding sensitized sheep cells, and the titres were taken as the highest serum dilution which gave 75% or more fixation of complement.

Neuraminidase inhibiting (NI) antibody tests. NI antibody tests were carried out by an automated method, based on the standard World Health Organization technique (Aymard-Henry *et al.* 1973), as described previously (Bevan, 1974).

Secretory IgA determination. Nasal washings were concentrated 10-fold by dialysis against 30% Carbowax, and the concentration of IgA was measured by the single radial diffusion method (Mancini, Carbonara & Heremans, 1965). Antiserum against human secretory IgA was obtained from Dakopatts, Copenhagen, Denmark, and a standard secretory IgA was prepared from human breast milk by block electrophoresis in Pevikon-Geon (Fahey & McLaughlin, 1963) and DE 52 cellulose chromatography.

Protein concentration

The concentration of protein in nasal washings was estimated in unconcentrated specimens by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Serum HI titres of the study group

From the original student volunteers, two groups each of 23 volunteers completed the study. The vaccinees were selected on the basis of the HI antibody titre to A/England/42/72 in serum specimens taken prior to immunization, and each volunteer was matched with a control volunteer with the same or similar serum HI antibody titre. A comparison of the two groups is shown in Table 1. In each group, 15 subjects had serum HI titres to influenza virus A/England/42/72 of $< 1/20$, four subjects had titres of 1/20–1/40 and four subjects had titres of $\geqslant 1/60$; the geometric mean titre (gmt) of serum HI titres were almost identical in the two groups. In assigning volunteers to the two groups, no account was taken of the pre-immunization titres of serum NI antibody.

Serum antibody response to immunization with A/England/42/72 vaccine

The serum HI and NI antibody response of the vaccinees to intramuscular immunization with 600 i.u. of A/England/42/72 surface antigen absorbed vaccine is shown in Table 2. Of the 15 students with serum HI antibody titres of $< 1/20$ before immunization, 14 developed HI antibody, and all the volunteers showed some increase in serum NI antibody titre after immunization; the gmt of serum HI and NI antibody increased from 1/5·4 to 1/196·3 and from 1/16·7 to 1/81·1, respectively. The serum HI antibody response to immunization was greater for the volunteers with some antibody before immunization. Thus, for the four volunteers with serum HI antibody titres of 1/20–1/40, the gmt of serum HI antibody increased from 1/29·1 to 1/1109, and for the four subjects with pre-immunization

Table 1. *Pre-immunization serum antibody titres of volunteers*

Group	No. tested	Serum antibody titres (A/England/42/72) HI (range)	HI (mean-gmt)
Controls	15	< 20	5·2
	4	20–40	29·3
	4	⩾ 60	108·2
Vaccinees	15	< 20	5·4
	4	20–40	29·1
	4	⩾ 60	108·4

Table 2. *Response of volunteers to immunization with A/England/42/72 surface-antigen-adsorbed vaccine*

Volunteer no.	Response to immunization: HI titre Ac. serum	HI titre Conv. serum	NI titre Ac. serum	NI titre Conv. serum
1	< 10	1920	< 20	60
2	< 10	120	60	240
3	< 10	< 10	< 20	320
4	< 10	60	< 20	120
5	< 10	640	< 20	60
6	< 10	60	< 20	30
7	< 10	240	< 20	80
8	< 10	1920	< 20	30
9	< 10	480	30	80
10	< 10	1280	30	60
11	< 10	30	< 20	120
12	< 10	60	20	80
13	15	120	30	40
14	< 10	240	40	120
15	< 10	640	NT	NT
Mean (gmt)	5·4	196·3	16·7	81·1
16	30	640	< 10	80
17	40	640	160	240
18	30	1920	< 10	30
19	20	1920	< 10	40
Mean (gmt)	29·1	1,109	11·9	69·0
20	60	3840	40	120
21	80	1920	< 10	120
22	120	1920	40	160
23	240	1920	30	640
Mean (gmt)	108·4	2,284	22·6	190

titres of serum HI antibody of ⩾ 1/60, the gmt increased from 1/108·4 to 1/2284 after immunization (Table 2). Increased titres of serum NI antibody were also found in these volunteers.

None of the students complained of adverse reactions to the vaccine; three volunteers reported a sore arm at the site of inoculation lasting a few hours, but

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Table 3. *Serum and nasal antibody response, and nasal protein and IgA concentrations in volunteers immunized with A/England/42/72 surface-antigen-adsorbed vaccine*

Serum HI antibody before immunization (no.)	Response to immunization: Nasal wash responses: Antibody production	Nasal wash responses: Mean protein (range, μg./ml.)	Nasal wash responses: Mean IgA (range, μg./ml.)	Serum antibody response: HI response (gmt)	Serum antibody response: NI response (gmt)
< 20 (15)	8 positive	381 (130–747)	34 (17–80)	< 20–515	16–77
	7 negative	211 (52–367)	22 (11–39)	< 20–399	20–91
⩾ 20 (8)	2 positive	370 (232–507)	33 (26–40)	49–1920	10–60
	6 negative	197 (62–367)	15 (5–26)	59–1494	30–145

these students qualified their comments by saying that the reaction was not severe enough to report, except in response to a direct question.

Local antibody response to immunization with A/England/42/72 vaccine

Nasal washings from volunteers immunized with A/England/42/72 surface-antigen-adsorbed vaccine were examined for homologous neutralizing antibody, protein and secretory IgA. None of the volunteers produced detectable nasal-wash neutralizing antibody before immunization, but antibody was found in nasal washings collected 2 and/or 3 weeks after immunization from ten subjects. The results are shown in Table 3. Eight of the 15 vaccinees who had no demonstrable serum HI antibody and two of eight volunteers with detectable serum antibody before immunization produced nasal wash antibody. The results indicate that a higher proportion of volunteers with no detectable serum HI antibody at the time of immunization developed nasal wash antibody, but the numbers were too small for statistical analysis.

Although the concentration of protein in nasal washings varied considerably for the different volunteers, the concentrations for the five samples from any single subject were similar; this was also true for the concentration of secretory IgA in nasal washings. For this reason, the concentration of protein and IgA in nasal washings has been averaged for each volunteer. The relation between the production of nasal-wash antibody, the concentration of protein and IgA in these specimens and the serum HI antibody response to immunization is shown in Table 3. For the eight volunteers with no detectable serum HI antibody before immunization, but who developed detectable nasal wash neutralizing antibody after immunization, the mean rise in serum HI antibody titre was from < 1/20 to 1/515 (gmt). In contrast, the remaining seven volunteers in this group who had no pre-immunization serum antibody and who did not produce detectable nasal antibody showed a mean serum HI antibody response to immunization of < 1/20 to 1/399.

Table 4. *Infection with MRC-7 virus in volunteers previously given A/England/42/72 surface-antigen-adsorbed vaccine*

Group	Serum HI antibody titre*	No. tested	Infection by MRC-8 virus: Virus isolation	Infection by MRC-8 virus: Signif. (X4) HI/CF antibody rise	Infection by MRC-8 virus: Total	Total (%)
Vaccinees	< 20	15	0/15	1/15	1/15	
	20–40	4	0/4	0/4	0/4	1/23
	⩾ 60	4	0/4	0/4	0/4	(4·3)
Controls	< 20	15	3/15	10/15	10/15	
	20–40	4	0/4	2/4	2/4	14/23
	⩾ 60	4	1/4	1/4	2/4	(61)

* Serum HI titres before immunization.

Thus, local antibody production was more common in volunteers with the greater serum HI antibody response. Eight vaccinees had serum HI antibody titres of ⩾ 1/20 before immunization; all produced high titres of serum antibody after immunization. The two volunteers in this group who developed nasal wash antibody after immunization showed the same serum HI antibody response as the remaining six who did not produce local antibody.

Local antibody production was more frequently observed in volunteers who produced relatively high concentrations of nasal wash protein and secretory IgA (Table 3). Thus, for the eight volunteers who had no detectable serum HI antibody before immunization but developed nasal wash antibody after immunization, the mean nasal wash protein concentration was 381 μg./ml. and the mean secretory IgA concentration was 34 μg./ml.; for the seven volunteers who did not produce detectable nasal wash neutralizing antibody, the mean protein and secretory IgA concentration in nasal washings was 211 μg./ml. and 22 μg./ml., respectively. A similar result was found for the volunteers who had serum HI antibody before immunization, but this group was too small to allow any conclusion (Table 3).

Response to challenge infection with MRC-7 virus

Four weeks after immunization the vaccinees, together with the control subjects, were each inoculated intranasally with $10^{7 \cdot 5}$ EID 50 of MRC-7 virus in a 1·0 ml. volume. The incidence of virus infection in the two groups of volunteers is shown in Table 4. The serum antibody response to immunization in the vaccinees gave high titres of serum HI antibody, but not CF antibody. In the present study, therefore, the CF test was used in addition to the HI tests to obtain serological evidence of infection by the challenge virus, since a fourfold rise in serum HI antibody as a measure of infection would have required a massive antibody response in immunized subjects.

Virus was not recovered from any of the 23 vaccinees given MRC-7 virus, and a fourfold rise in serum antibody was measured in only one subject. The single individual in this group who showed serological evidence of infection with the challenge virus had responded relatively poorly to the vaccine (< 1/20 to 1/30),

and had not produced local antibody after immunization. In contrast to the results of challenge infection in immunized volunteers, evidence of infection with MRC-7 virus was found in a greater number of control subjects (Table 4). Thus, virus was recovered three days after infection and a significant serum antibody response was found in three subjects, serological evidence of infection was obtained for a further 12 volunteers, and virus was recovered from a single subject who showed no serological rise in serum antibody titre. In total, evidence of infection was found in 14 of 23 (61%) of the control group, as compared with one of 23 (4·3%) immunized volunteers (Table 4).

DISCUSSION

The serum antibody response of volunteers to immunization with influenza virus subunit vaccines has varied considerably, depending on the method employed to prepare the vaccine. A virus haemagglutinin vaccine was reported to induce serum antibody to titres comparable with those produced by whole virus vaccine (Webster & Laver, 1966; Davenport *et al.* 1964; Hennessey & Davenport, 1974), and similar results were obtained from tri (n-butyl) phosphate-split vaccine (Neurath, Rubin, Sillaman & Tint, 1971; Ruben & Jackson, 1972). Studies in mice have shown that tween-ether treated virus vaccine was more antigenic than whole virus (Fenters, Yamashiroya, Petzold & Tolkacz, 1970). In contrast, DOC-split vaccine was less antigenic than whole virus (Webster & Laver, 1966), and the highly purified virus haemagglutinin vaccine produced by bromelain treatment of virus particles (Brand & Skehel, 1973) was found to be a poor inducer of serum antibody in ferrets (McLaren, Potter & Jennings, 1974).

Although split-virus vaccines vary in immunogenicity, a more uniform result has been obtained in studies of the toxicity of these products. Thus, virus haemagglutinin vaccines, ether-tween split vaccine, and DOC-split vaccine have all been found to produce less reactions than whole virus vaccine (Brandon *et al.* 1967; Zavadova *et al.* 1972; Webster & Laver, 1966). The tri (n-butyl) phosphate-split vaccine was also found not to produce significant reactions in volunteers inoculated with concentrations of 6400 CCA (Ruben & Jackson, 1972), but a further study with this vaccine reported reactions to high titres of vaccine in subjects with relatively high homologous serum HI antibody titres, possibly due to a hypersensitivity reaction (Ruben, Potter & Stuart-Harris, 1975). Recent studies have shown that much of the toxicity associated with whole virus vaccine may be due to contamination with non-virus material; reactions were significantly reduced for whole virus vaccines purified by zonal centrifugation (Kilbourne *et al.* 1974).

The present study was carried out using a vaccine designed with consideration for both the known toxicity and immunogenicity of existing influenza vaccines. Virus particles were purified by zonal centrifugation and subsequently treated with Triton N101 and purified to give a preparation of virus haemagglutinin and neuraminidase. Although it would be expected that this surface antigen product was free of toxicity, the material was much less immunogenic in hamsters than purified whole virus (Jennings, Potter, McLaren & Brady, 1975). In addition, tests in hamsters showed that even in the presence of serum antibody induced by the

surface antigen material in saline, the animals were susceptible to homologous challenge infection (Jennings *et al.* 1975). The adsorption of the purified haemagglutinin and neuraminidase to an alhydrogel carrier significantly increased the immunogenicity of the vaccine; this approach was not new, since purified subunit vaccine obtained by ether extraction and adsorption to $AlPO_4$ was found to have an enhanced antibody-inducing capacity for mice but not for man, compared with unadsorbed vaccine (Davenport, 1968; Davenport, Hennessy & Askin, 1968). The present vaccine induced higher titres of serum antibody in hamsters than either the non-adsorbed virus subunits or the whole virus from which it was derived, and produced immunity to the challenge infection in hamsters comparable with that found in animals immunized with whole virus (Jennings *et al.* 1975).

The results of the present study indicate that the surface-antigen-adsorbed vaccine produced no significant toxic reactions in a small group of healthy volunteers, induced relatively high titres of both serum HI and NI antibodies, and conferred highly significant protection against challenge infection. By removing the probably immunologically irrelevant and toxic core material, and adsorbing the purified surface antigens to an inert carrier to promote the immune response, the vaccine may be considered to be a logical development of an inactivated vaccine against influenza.

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