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An adsorbed surface-antigen influenza vaccine and its serological activity in volunteers

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Summary

A highly purified vaccine prepared from surface antigens of influenza virus adsorbed on to aluminium hydroxide has elicited good antibody results to both haemagglutinin and neuraminidase components, and has been shown to induce resistance to homotypic challenge at a satisfactorily high level.

Introduction

The purification of influenza virus vaccine has been facilitated by two major technological advances, namely the development of zonal rotors capable of processing large volumes of fluid, and the application of surfactants, lipid solvents or proteases to disrupt the influenza virion. However, application of either of these two principles can still leave one with a vaccine which contains large amounts of matrix and nuclear proteins, which do not appear to contribute to the protective efficacy of the vaccine and are probably responsible for at least some of the local and systemic reactions occurring in vaccinated subjects.

In general, however, disruption of the virus particle is accompanied by a loss of antigenicity. The more efficient the breakdown and the more stringent the purification of selected protein fractions, the greater tends to be the overall loss of antigenicity. The surface proteins, the haemagglutinin and neuraminidase, are of relatively low molecular weight and apart from actual process losses of antigenic mass, the separated antigens appear to have less potential than does the same amount of material in the intact particle. This problem can be readily overcome by the incorporation of a mineral carrier such as aluminium hydroxide.

A vaccine consisting of haemagglutinin and neuraminidase antigens adsorbed on to aluminium hydroxide gel ('Alhydrogel') was prepared and the dose response to 0·5 ml doses containing different amounts of antigen was studied in volunteers.

Materials and methods

Virus strains

A/England/42/72 and A/Port Chalmers/1/73 strains of influenza virus were obtained from the Medical Research Council Influenza Reference Laboratories, Mill Hill, London.

Virus cultivation

Influenza virus ($10^{4\cdot0}EID_{50}$) was inoculated by the allantoic route into 11-day-old embryonated hen eggs (0·2 ml/egg). The eggs were incubated at 35°C for 48 hr. They were chilled at 4°C and the allantoic fluid was harvested. Beta-propiolactone was added to the final concentration of 0·1% to inactivate the virus.

Purification of whole virus

Allantoic fluid (140 l) containing virus was clarified at 15,000 *g* in an Alfa-Laval centrifuge to remove gross contaminants, e.g. erythrocytes. A gradient was established in a KII zonal rotor by adding sucrose (60% w/w 1·4 l) to the stationary rotor and slowly adding 1·8 l of phosphate buffered saline (PBS) (0·01 mol/l phosphate pH 7·6 in physiological saline).

The gradient was formed by centrifugation at 3000 r.p.m. for 15 min. The rotor was accelerated and a fast flow of PBS (20–30 l/hr) was started. When the rotor reached a speed of 35,000 r.p.m. (90,000 *g*) the PBS was replaced with clarified allantoic fluid at a flow rate of 30 l/hr. Under these conditions virus particles entered the sucrose gradient and formed a band at their isopycnic density. When all the virus had been centrifuged across the gradient, the rotor was operated at 90,000 *g* for a further hour at room temperature.

At the end of centrifugation, when the rotor slowed to a halt, the gradient reorientated. Fractions were collected and those containing a high concentration of virus were pooled and diluted to 5% w/w sucrose with PBS to a final volume of 10 l.

Disruption of virus

A gradient was established in the KII rotor by adding PBS (1·8 l) containing Triton N101 (1% v/v) to sucrose (60% w/w in PBS) (1·4 l) also containing

Triton N101 (1% v/v). The gradient formed as the rotor accelerated to 90,000 *g* and the Triton micelles formed a band within minutes.

Purified virus diluted to 10 l as described above was allowed to flow across this gradient and disruption occurred as the virus particles sedimented through the band of Triton. The rotor was operated for a further hour at room temperature and 90,000 *g* to separate the split products, the surface antigens sedimenting in the Triton layer, while the core and matrix proteins settled in the denser portion of the gradient.

Removal of Triton

The fractions from the gradient which contained surface proteins were pooled and phosphate buffer (2 mol/l pH 7·0) was added to give a final concentration of 0·5 mol/l. Under these conditions, the cloudpoint of the surfactant was exceeded at room temperature. The solution was allowed to stand at room temperature overnight and the Triton separated as a narrow upper layer, which also contained phospholipids. The lower phase containing the surface proteins of the virus was removed.

Adsorption of surface antigens to aluminium hydroxide

Alhydrogel (Superphos Ltd) (2% w/v $Al(OH)_3$ in water) was added to the concentrated surface antigens after removal of Triton. The suspension was allowed to stand overnight at 4°C and then diluted to the required strength. Vaccines were prepared containing 400 i.u./0·5 ml, 200 i.u./0·5 ml, 100 i.u./0·5 ml, 50 i.u./0·5 ml and 25 i.u./0·5 ml. The final vaccine contained $Al(OH)_3$ (5 mg/ml) in 0·1 mol/l phosphate buffer.

Standardization of vaccine

The haemagglutinin content of the vaccine was standardized by radial diffusion (Schild, Henry-Aymard and Pereira, 1972) using antiserum prepared in rabbits to bromelain-derived haemagglutinin (Brand and Skehel, 1972). Neuraminidase was estimated by direct assay of enzyme activity in an autoanalyser (Bevan, Furminger and Smith, 1975).

Electron microscopy

Dilute samples were concentrated by sedimentation and resuspended in PBS. Samples were added to carbon-coated formvar grids and salts were removed by addition and removal of distilled water to the grid. Phosphotungstic acid (2·5% w/v) pH 6·8 was used as a negative stain.

Results

Figure 1 shows the distribution of viral components after virus particles have been centrifuged through a sucrose gradient containing Triton N101. The protein peak at the dense end of the gradient represents the stripped particles or cores and some residual unsplit virus as indicated by the small neuraminidase peak. The protein at the top of the gradient consists of haemagglutinin and neuraminidase close to the surfactant band.

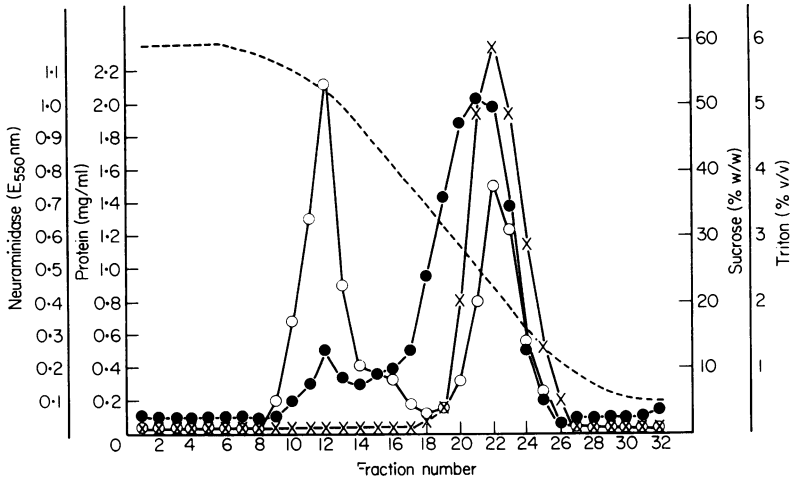

FIG. 1. Distribution of A/England/42/72 influenza proteins after centrifugation through a sucrose gradient containing Triton N101. Levels of protein, ○—○; neuraminidase, ●—●; Triton N101, X—X; sucrose, - - - - are shown for each fraction.

FIG. 2. Surface antigens of A/England/42/72 showing the cartwheel formations of neuraminidase and the stellate aggregates of haemagglutinin.

Figure 2 shows the effect of removing surfactant from a pool of the fractions containing haemagglutinin and neuraminidase. The two surface proteins form separate aggregates and the stellate groups of haemagglutinin particles contrast strongly with the cartwheel formations of neuraminidase. No particles of core protein were observed and specific testing with core protein antibody by radial diffusion gave a negative reaction (Oxford, 1974).

Serological studies

For studies in volunteers, serial two-fold dilutions of material prepared from the A/Port Chalmers/1/73 strain were made in aluminium hydroxide gel so that the concentration of gel was constant at 2·5 mg per dose (= 0·86 mg aluminium) at each dose level. Subjects with no recent history of influenza-like illness and who had not received influenza vaccine within the previous 12 months were bled and their serum HI (haemagglutination inhibition) and NI titres determined. They were then distributed into groups so that the numbers with low and high HI antibody levels were evenly matched. The diluted vaccines were coded and each group was given a different dilution on a random basis. Two to three weeks later a further blood sample was taken and antibody levels again determined.

The results are shown in the following tables, the titres being shown as reciprocal values in each case. If the subjects with low HI antibody levels are considered (Table 1) there appears to be no dose response curve, even 25 i.u. producing a good level. If all subjects are considered (Table 2) there is a suggestion of a lower level of response at doses of 50 and 25 i.u. but the differences are not statistically significant.

TABLE 1. Haemagglutination inhibition responses of volunteers with low antibody levels (< 1 : 30) to A/Port Chalmers surface antigen vaccine

Dose (i.u.)	Seroconversion rate	g.m.t.* Pre-vaccination	g.m.t.* Post-vaccination
400	8/ 8	3	384
200	8/ 9	4	396
100	13/15	3	331
50	6/ 7	5	234
25	5/ 6	2	384
All doses	40/45	3	295

* Geometric mean titre

TABLE 2. Haemagglutination inhibition (HI) responses of volunteers to A/Port Chalmers surface antigen vaccine (all subjects)

Dose (i.u.)	Seroconversion rate	g.m.t. Pre-vaccination	g.m.t. Post-vaccination
400	11/12	11	543
200	10/11	6	612
100	22/24	13	550
50	9/10	13	384
25	9/10	13	384
All doses	61/67	11	501

With neuraminidase antibody, on the other hand (Table 3) there is a very marked fall in response to doses below 200 units.

TABLE 3. Neuraminidase antibody responses to different doses of A/Port Chalmers neuraminidase

Dose (arbitrary units of activity) (i.u.)	Seroconversion rate	g.m.t. Pre-vaccination	g.m.t. Post-vaccination
400	10/12	<5	15·5
200	10/11	<5	15·9
100	15/24	<5	11·2
50	8/10	<5	8·3
25	3/10	<5	2·4

It has been suggested that subunit vaccines produce a narrower HI response than do whole virus vaccines. This does not, however, seem to be the case with our adsorbed preparations, since the spectrum of antibody response in six subjects, all of whom had an HI titre of 1 : 12 before vaccination, was broad (as seen in Table 4).

Table 5 shows the antibody response in elderly subjects to purified surface antigen vaccine, and although it does not appear to be as good as in young adults, it is adequate. Table 6 shows the results of a challenge study in student volunteers who received 400 i.u. of a surface antigen vaccine prepared from the A2/Eng/42/72 strain and were subsequently challenged with the attenuated MRC-7 recombinant. As can be seen, the incidence of a successful challenge in the control group was 57% compared with approximately 4% in the vaccinated subjects.

TABLE 5. Homologous haemagglutination inhibition (HI) response in elderly subjects to A/Port Chalmers surface antigen vaccine. Twenty-eight subjects aged 65–76 (mean 71·3) years

Pre-vaccn HI titre	Sero conversion	g.m.t. Pre-vaccination	g.m.t. Post-vaccination
<20	19/22	<20	127
30–120	5/ 6	38	1599
Total	24/28 (86%)	<20	218

Discussion

Previous studies in ferrets (Brady and Furminger, 1975) had shown that a vaccine prepared from purified influenza haemagglutinin and neuraminidase was effective in protecting these animals against challenge infection with the homologous strain of virus. It was therefore of interest to investigate the antibody response and reactogenicity in human subjects and the response of volunteers to direct challenge with an attenuated virus.

The lack of any general reactions and the absence of any reports of severe local reactions to the surface

TABLE 4. Heterologous antibody response to haemagglutinin of A/Port Chalmers/73. Six subjects all with A/Port Chalmers titre of < 1 : 12 pre-vaccination

Subject	Viruses A/Scot/74	A/S.Aust/74	x31	A/42/74	A/PC
A.B.	384	96	384	192	96
B.Y.	3072	768	>6144	3072	6144
M.B.	768	192	>6144	1536	384
M.S.	48	24	96	24	48
M.C.	>6144	3072	>6144	>6144	>6144
J.R.	48	24	96	192	24

TABLE 6. Infection with MRC-7 virus in volunteers previously given A2/Eng/42/72 vaccine, and in controls

Group	Serum HI antibody titre*	No. tested	Infection with MRC-7 by: Virus isolation	Significant (x4) HI antibody response	Total	Total (%)
Vaccinees	<20	15	0/12	1/15	1/15	1/23 (4·3%)
	20–40	4	0/2	0/4	0/4	
	>60	4	0/3	0/4	0/4	
Controls	<20	15	3/11	10/15	10/15	13/23 (57%)
	20–40	4	0/4	2/4	2/4	
	>60	4	0/4	1/4	1/4	

* Serum HI antibody titres before immunization.

antigens suggests that the further purification involved in removing core and matrix proteins has resulted in a more satisfactory vaccine. The additional purity may also account for the flatness of the dose response curve to the haemagglutinin, by minimizing the extent of antigenic competition.

A similarly flat dose response has been observed in chickens, but the bird-to-bird variation makes this species an unsatisfactory test animal for standardization of the vaccine.

Further studies are in progress to assess the duration of the antibody response and to study the response of volunteers to challenge infections after graded doses of antigen.

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