Jl. Hyg., Camb. (1979), **82**, 51
With 1 plate
Printed in Great Britain

The specificity of the anti-haemagglutinin antibody response induced in man by inactivated influenza vaccines and by natural infection

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(*Received* 5 *April* 1978)

SUMMARY

The anti-haemagglutinin antibody response in adult human volunteers to inactivated whole virus or tween ether split influenza A/Victoria/75 (H3N2) and A/Scotland/74 (H3N2) virus vaccines was investigated using antibody adsorption and single-radial-haemolysis (SRH) techniques. The concentrations of haemagglutinin (HA), nucleoprotein (NP) and matrix (M) antigens measured by single radial diffusion (SRD) and rocket immunoelectrophoresis were similar for both the whole virus and split vaccines. Whole virus and split vaccines induced cross-reactive (CR) antibody in 87% of vaccinees. Strain specific (SS) antibody to A/Hong Kong/1/68 or the homologous virus was induced less frequently than CR antibody. Higher anti-haemagglutinin antibody titres were detected in persons receiving the split virus vaccines than in those receiving the whole virus vaccines. No antibody to the type-specific matrix protein was detectable, but 33% of volunteers developed an antibody rise to type-specific nucleoprotein antigen.

The specificity of the anti-haemagglutinin antibody response in human adults to natural infection with A/Port Chalmers/73 (H3N2) virus was similar to that induced by inactivated vaccines in that a high proportion of subjects developed CR anti-haemagglutinin antibody, which reacted with A/Hong Kong/68 virus and the homologous A/Port Chalmers/73 virus, and SS antibody for A/Hong Kong/68 virus but SS antibody for A/Port Chalmers/73 virus was infrequently stimulated by natural infection.

INTRODUCTION

The haemagglutinin molecule of influenza A viruses is a triangular spike of dimensions approximating to 5–7 nm width and 11–16 nm length (Laver & Valentine, 1969; Nermut, 1972; Schultz, 1972) and molecular weight of 210000 daltons. Such a molecule would be expected to have multiple antigenic determinants and to date two groups of determinants have been described (Virelizier *et al.* 1974*a*; Virelizier, Allison & Schild, 1974*b*; Laver, Downie & Webster, 1974).

0022–1724/79/0044–1978 $01.00

The HA molecules of influenza A viruses of the A/Hong Kong/68 subtype share common antigenic determinants which have been designated as cross-reactive (CR) determinants (Schild *et al.* 1974). The HA molecules of antigenically variant viruses within the A/Hong Kong subtype also possess unique strain specific (SS) antigenic determinants. In view of the suggestion (Virelizier, 1975) based on experimental studies in mice that anti-HA antibodies of CR and CS specificity may differ in their protective values it is important to establish the specificity of anti-HA response in man induced by inactivated vaccines. We now report that inactivated influenza A/Victoria/3/75 vaccines, either as whole or split virus, infrequently induced in adult recipients SS antibody to the HA of the homologous virus but frequently induced CR antibody which reacted with the earliest epidemic strain of the subtype, A/HK/68 virus and the homologous virus used in the vaccine, and also induced SS antibody to A/HK/68 virus.

MATERIALS AND METHODS

Viruses

Influenza viruses A/Victoria/3/75 (H3N2), A/Scotland/540/74 (H3N2), A/Port Chalmers/73 (H3N2), A/Hong Kong/1/68 (H3N2) were grown in embryonated hens' eggs by standard procedures. Virus was concentrated and purified as described previously (Skehel & Schild, 1971).

Inactivated whole virus or split virus vaccines

Inactivated influenza virus vaccines were prepared by Philips-Duphar B. V., Weesp, Holland. Influenza viruses A/Victoria/75 and A/Scotland/74 were grown in embryonated eggs, purified by zonal centrifugation and inactivated with β-propiolactone. Some samples of these whole virus preparations were then split by treatment with tween (0·01 %) and ether at 4 °C for 18 h, the water-phase was collected and twice further extracted with ether. The final water phase material was dialysed extensively against phosphate buffered saline (PBS) containing 0·01 % merthiolate. Assay of the haemagglutinin content of the vaccines by single-radial-diffusion (Wood *et al.* 1977) indicated that the monovalent whole virus A/Scotland/74 and A/Victoria/75 vaccines contained 45 and 21 μg HA per 0·5 ml dose respectively. The split virus A/Scotland/74 and A/Victoria/75 vaccines contained 29 and 16 μg HA per 0·5 ml dose respectively. Both whole virus and split virus A/Scotland/74 and A/Victoria/75 vaccines contained NP antigen. When assayed by rocket immunoelectrophoresis (Oxford & Schild, 1977) 30–40 μg NP per 0·5 ml dose was detected for each of the vaccines.

Study design

Two separate studies were carried out in student volunteers in 1976 using the same experimental design which has been described in detail elsewhere (Jennings *et al.* 1978). The antigenicity of whole and split vaccines containing A/Scotland/74 virus was compared in one study, and of A/Victoria/75 whole and split virus in the second study.

Single radial haemolysis

Ten per cent (v/v) suspensions of freshly washed sheep erythrocytes (Oxoid) were made up in physiological saline buffered with 0·05 M HEPES buffer pH 6·5. Chromium chloride was freshly diluted 1/400 in physiological saline from a 2·25 M solution (Vaananen *et al.* 1976). Influenza virus, in the form of clarified allantoic fluid or purified virus was added at the rate of 10000–12000 haemagglutinating units (HAU) or 100 μg virus protein per ml of 10% erythrocyte suspension. Within 10 min at 4 °C visible haemagglutination occurred and a half volume of the freshly diluted $CrCl_3$ solution was then added. The mixture was allowed to stand at room temperature for 5 min with occasional mixing. The cell suspension was sedimented by gentle centrifuging (100 rev/min for 5 min), washed once in 0·05 M HEPES pH 6·5 buffer containing 0·2% (w/v) bovine serum albumin (BSA), once in phosphate buffered saline (PBS) pH 7·2 containing 0·2% BSA and finally re-suspended to 10% in PBS without BSA. Immunoplates containing virus sensitized cells and fresh guinea-pig complement in agarose gel were prepared as described by Schild, Oxford & Virelizier (1976). The prepared plates could be stored at 4 °C for several weeks before use.

Adsorption experiments to determine anti-HA antibody specificity

Analysis of the CR and SS antibody content of human sera was carried out using techniques described previously (Schild *et al.* 1977). In summary, paired sera taken before and after immunization were analysed for anti-HA antibody specificity in SRH plates containing A/HK/68 virus, A/Scotland/74 virus or A/Victoria/75 virus. Undiluted serum (40 μl) was heat inactivated (56 °C for 30 min) and incubated for 30 min at room temperature with 10 μl of saline or 10 μl of A/HK/68 viruses (purified virus containing 10–15 mg/ml protein) or 10 μl of either A/Scotland/74 or A/Victoria/75 viruses. Twenty μl volumes of the virus-serum mixture were then added to the wells of an SRH plate containing A/HK/68 virus or A/Victoria/75 or A/Scotland/74 virus (Pl. 1). Previous tests had established that an excess of virus was used in these experiments which would adsorb completely either SS or CR antibody. SRH plates were incubated overnight at 37 °C and the lysis zones measured using a calibrating viewer (Transdyne General Corporation). Representative results are illustrated in Plate 1. Thus comparison of the pre- and post-vaccination sera from a subject (Pl. 1A, top row) showed a small rise in anti-HA antibody when the unadsorbed sera were tested in an SRH plate containing A/Victoria/75 virus. The sera reacted similarly on a plate containing A/HK/68 virus (Pl. 1C). For the pre-immunization serum, zones were of similar size on the A/Victoria/75 and A/HK/68 plates and the antibody was adsorbed by the heterologous virus. This indicated that the antibody in the pre-immunization serum was predominantly CR in specificity. Antibody in the post-immunization serum detected on a plate containing A/Victoria/75 virus was not completely adsorbed by A/HK/68 virus (Pl. 1A, second row), a procedure which would remove all CR antibodies. Thus a proportion of the anti-HA antibody molecules in the serum reacted with

SS antigenic determinants of the homologous virus, A/Victoria/75 (SS_{Vic}). After adsorption of the same post-immunization serum with A/Victoria/75 virus no lysis zone was detectable because of the removal of both CR and SS antibodies (Pl. 1A, third row). The same serum pair was tested on an SRH plate containing A/HK/1/68 virus and showed an increase in the number of antibody molecules reacting with antigenic determinants of the homologous virus. Since these antibodies were completely adsorbed by A/HK/68 virus (Pl. 1C, second row) but not absorbed by A/Victoria/75 virus (Pl. 1C, third row) it was concluded that the antibodies were reacting with SS determinants of A/HK/68 virus (SS_{HK}). Thus in this subject immunization with A/Victoria/75 vaccine induced antibodies which reacted with the SS determinants of both A/Victoria and A/HK/68 virus in addition to CR antibodies. In the serum pair illustrated in Pl. 1B and D, the pre-immunization serum contained SS_{HK} antibody and no CR antibody. The post-immunization serum contained CR antibody and an unchanged quantity of SS_{HK} antibody.

Assay of antibodies to influenza nucleoprotein and matrix protein antigen

Avian virus A/chicken Germany/'N'/49 (Hav2Neq1) which contained surface HA and NA antigens for which there is no antibody present in human sera but which contained NP and M antigens closely related to those of human influenza A viruses, was disrupted with 1% sodium sarcosyl (SS, Ciba-Geigy NL97) and incorporated in agarose in single radial diffusion plates (Oxford & Schild, 1976). For the detection of antibodies to M and NP virus was used at a final concentration of 150 μg protein/ml agarose. In such SRD plates zones attributed to M antibody were of small diameter and dense, whilst zones of NP were larger and less intense (Mostow *et al.* 1975). To detect antibodies to M antigen specifically, virus was disrupted with 1% (v/v) SS, boiled for 2 min, and incorporated in immunoplates at a concentration of 20 μg/ml. The type-specific determinants of M protein are stable to heating whereas the other antigens of the virus are denatured (Oxford & Schild, 1976). The SRD plates were incubated overnight at room temperature and the diameter of the precipitin zones measured as described above.

RESULTS

The SRH test using chromium chloride to attach virus to the red blood cells was a sensitive and rapid method for the detection of specific antibody to influenza virus HA.

Detailed analysis of paired sera from volunteers receiving inactivated whole or split virus vaccines are presented in Table 1. Similar results were obtained for both A/Scotland/74 and A/Victoria/75 vaccines. Following vaccination with either whole or split A/Victoria/75 vaccines, the majority (89–94%) of vaccinees responded by producing CR antibody. A low proportion (17–26%) developed SS_{HK} antibody or SS_{Vic} antibody (17–21%). No significant difference in the specificity of antibody produced was detected in persons given whole or split vaccines. Only 4 of 19 (21%) persons immunized with whole A/Victoria/75 vaccine and 3 of 18

Table 1. *Specificity of HA antibody produced after immunization with inactivated influenza vaccine*

		No. of vaccinees with following antibody										
		SS_{HK}			SS_{Vic} or Scot			CR			SS + CR	
Vaccine	Total no. of vaccinees	Pre	Post	% rises	Pre	Post	% rises	Pre	Post	% rises	Pre	Post
A/Scotland/74												
Whole virus	12	9	11 (1)	8·3	2	4 (3)	25	10	12 (10)	83	8	11
Split virus	18	17	17 (5)	28	1	3 (3)	17	15	18 (15)	83	14	17
A/Victoria/75												
Whole virus	19	15	17 (5)	26	3	4 (4)	21	7	19 (17)	89	7	17
Split virus	18	14	14 (3)	17	1	3 (3)	17	9	18 (17)	94	7	14

( ) No. of vaccinees with a significant rise in antibody titre. SS_{HK}, strain-specific antibody to HA of A/HK/1/68 (H3N2).
CR, cross-reactive antibody. Pre, post, serum taken pre- and post-immunization.

Table 2. *Quantitation of anti-HA antibodies in pre- and post-vaccination sera by SRH*

Vaccine	Mean area of haemolysis zones (mm^2)	
	Pre-immunization	Post-immunization
A/Scotland/74		
Whole virus	2·01	15·83
Split virus	6·13	32·52
A/Victoria/75		
Whole virus	0	19·35
Split virus	0	33·65

Table 3. *Antibody to influenza type specific nucleoprotein (NP) antigen produced after vaccination*

Vaccine	Total no. of vaccinees	No. of vaccinees with NP antibody		No. and % significant antibody rise
		Pre-vaccination	Post-vaccination	
A/Scotland/74				
Whole virus	17	6	10	8 (47%)
Split virus	19	12	16	5 (26%)
A/Victoria/75				
Whole virus	22	11	14	6 (27%)
Split virus	21	7	13	7 (33%)

(17%) persons immunized with split vaccine developed SS_{Vic} antibody. Before vaccination most persons had either CR antibody or SS_{HK} antibody or both, suggesting prior immunological experience with A/HK/68 virus or a closely related variant.

Measurement of the total anti-HA antibody response on SRH plates containing the homologous virus used in the vaccine (Table 2) indicated that similar quantities of anti-HA antibody were produced following immunization with split virus A/Scotland/74 and A/Victoria/75 vaccines with mean zone areas of 32·5 and 33·7 mm^2 respectively. However, the post-vaccination antibody titres were higher in recipients of the split virus vaccines than in the recipients of the whole virus vaccines.

Both whole and split virus vaccines contained NP antigen as demonstrated by rocket immunoelectrophoresis and therefore antibody responses to this antigen were investigated (Table 3). A high proportion of volunteers had serum antibody to NP antigen before vaccination. No significant differences were detected in the ability of whole virus or split virus vaccine to induce antibody to the NP antigen. No antibody to M protein was detected in these sera either before or after immunization.

For comparison with antibody responses following vaccination described above we analysed the antibody specificity following natural infection of young adults

https://doi.org/10.1017/S0022172400025468 Published online by Cambridge University Press

with influenza A/Port Chalmers/73 virus (Table 4). One of 17 persons developed SS antibody reacting with the SS determinant of A/Port Chalmers/73 virus, while 7 (41%) persons developed SS_{HK} antibody and 16 (94%) persons developed CR antibody reacting with A/HK/68 and A/Port Chalmers/73 viruses. Thus, the specificity of the anti-HA response following natural infection, in terms of CR and SS antibodies, was similar to that following vaccination.

DISCUSSION

The results demonstrated that following immunization with inactivated influeza vaccines containing either intact or split virions most adults responded by producing CR antibody. In contrast, a relatively low proportion of volunteers developed SS antibody reacting either with the first member of the subtype A/HK/68 or with the homologous virus. We detected an essentially similar response following natural influenza infection, with a low proportion of individuals producing SS antibody to the infecting virus. CR or SS antibody reacting with A/HK/68 was detected in the majority of sera both pre-immunization and pre-infection. This finding indicated that many individuals in the study group had been previously infected with A/HK/68 virus or a closely related strain which would have resulted in immunological priming to the A/HK/68 haemagglutinin. The present findings may have important implications for the formulation of inactivated vaccines. Experimental studies have shown that SS antibody is more efficient in conferring passive immunity in experimentally infected mice than is CR antibody (Virelizier, 1975). Vaccines inducing high titres of SS antibody in the majority of vaccinees might therefore be expected to give better protection against homologous influenza infection. The incomplete protection induced by currently available inactivated vaccines (reviewed in Selby, 1976) might in part be explained by the comparative failure of these vaccines to induce antibody to the SS determinant of the haemagglutinin. However, the results should not be interpreted as indicating that updating of the composition of influenza vaccines, so as to include the most recent antigenic variant of epidemiological importance, is unnecessary. Indeed, a proportion of volunteers developed SS antibody to the homologous virus in the vaccine. Furthermore, clinical studies have demonstrated that maximum protection is induced by vaccines containing viruses antigenically most close to the virus causing the epidemic (Larsen *et al.* 1978; Potter *et al.* 1977). Although in the present study using SRH techniques relatively few individuals appeared to develop SS antibody to the homologous virus in the vaccine it is possible that low titres of SS antibody are induced which might be detectable by more sensitive assay methods.

Recently Schild *et al.* (1977) reported that recipients of graded doses of inactivated whole virus A/Port Chalmers/73 vaccine mostly developed CR antibody reacting with both A/HK/68 and A/Port Chalmers/73 viruses. Only a low proportion of persons developed antibody to the SS determinants of the homologous A/Port Chalmers/73 virus. Similar findings using other techniques have been reported for A/Port Chalmers/73 vaccines by Webster *et al.* (1976).

Table 4. *Specificity of antibody response in persons naturally infected* with A/PC/73 (H3N2) virus*

No. of persons	No. of persons with following antibody									
	SS_{HK}			SS_{PC}			CR			SS + CR
	Pre	Post	% rises	Pre	Post	% rises	Pre	Post	% rises	Pre Post
17	12	12 (7)	41	0	1 (1)	6	10	17 (16)	94	10 12

* Serum samples were taken at the acute phase of the illness and 10–14 days post-infection.
( ) No. of persons with a significant rise in antibody titre.

https://doi.org/10.1017/S0022172400025468 Published online by Cambridge University Press

Earlier studies in man demonstrated the phenomenon of original antigenic sin (Francis, Davenport & Hennessey, 1953) and experimental studies in animal models have amplified these results and provided an explanation in terms of molecular immunology (Virelizier *et al.* 1974*a*, *b*). Most vaccinees in the present study were young adults and possessed antibody and/or immunological memory to the SS and CR determinants of A/HK/68 virus before the study began. Individuals possessing antigenic memory to the SS or CR determinants of A/HK/68 virus may be unable to respond or able to respond only poorly to produce antibody to the SS determinant of another variant. A large glycoprotein molecule such as influenza haemagglutinin would be expected to have many antigenic determinants and individuals may vary according to their responsiveness to these different determinants.

The present studies have shown that the specificity of anti-HA antibodies induced in volunteers by whole virus or split influenza vaccines was similar. Thus, the nature of the anti-HA antibody response did not appear to be greatly influenced by the physical rearrangement of the HA molecules in the vaccine induced by chemical treatment during the splitting of the virus. The HA antigen in the split virus vaccine used in these studies (Jennings *et al.* 1978) was essentially in the form of 'rosettes', each containing large numbers of HA 'spikes', rather than as isolated HA molecules. In addition virus 'core' material free of HA antigen was abundant in the split vaccine. However, the split virus vaccines induced higher titres of anti-HA antibody than whole virus vaccines containing comparable amounts of HA antigen. Whole virus and split vaccines evoked antibody to the NP antigen equally well, and both failed to induce antibody to M protein. The frequency of stimulation of antibody to NP antigen and the absence of stimulation of antibody to M protein by vaccines observed in the present study was similar to that reported by Mostow *et al.* (1975) in studies of inactivated whole virus vaccines in the USA. Experimental studies in animals have indicated that immunization with M or NP antigens is not protective against subsequent influenza infections (Oxford & Schild, 1976).

Experimental studies to investigate methods of presentation of influenza antigens in animals, or human volunteers, so as to increase the frequency and titre of antibody to the strain specific determinants of the HA antigen appear to be relevant to the problem of improving the efficacy of inactivated influenza vaccines. Such studies include the investigation of adjuvants and the coupling of HA to liposome or protein carriers.

We would like to thank Mrs T. Corcoran and Barry Watts for excellent technical and photographic assistance, and Philips-Duphar B. V., Weesp, Holland, for supplying the vaccines used in the present study.

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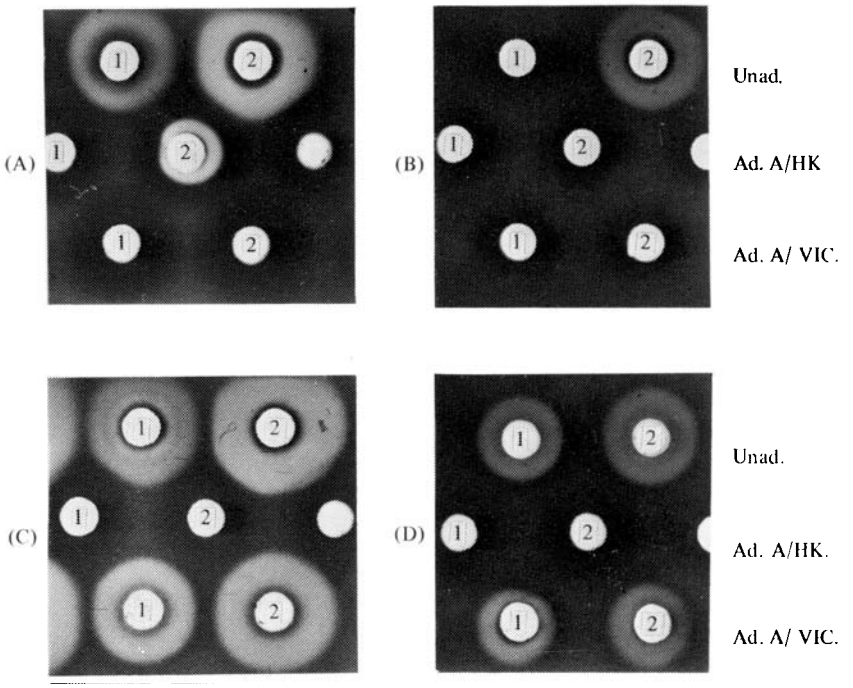

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https://doi.org/10.1017/S0022172400025468 Published online by Cambridge University Press

EXPLANATION OF PLATE

Plate 1

Analysis of representative sera by single radial haemolysis. (A), (B) SRH plates containing A/Victoria/75 (H3N2) virus. (C), (D) SRH plate containing A/HK/68 (H3N2) virus. 1 and 2 are pre- and post-immunization sera in each case.

Top row in each plate shows haemolysis reactions with unadsorbed sera. Second and third rows in each plate sera are adsorbed with A/HK/68 virus and A/Victoria/75 viruses respectively.

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