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Immunity Following Intranasal Administration of an Inactivated, Freeze-Dried A/England/42/72 Vaccine

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Summary

A group of 23 student volunteers were each inoculated intranasally with 400 IU of inactivated, freeze-dried A/England/42/72 vaccine. Only one volunteer showed a four-fold rise in serum HI antibody following immunization, and the mean increase in serum HI antibody (gmt) for all volunteers did not increase two-fold. Thirteen of the volunteers developed detectable levels of nasal wash neutralizing antibody after immunization; local antibody was most commonly found in volunteers who also produced a detectable but less than four-fold rise in serum antibody titre, and who produced nasal washings with relatively high concentrations of protein and secretory IgA. Four weeks after immunization, the vaccinees and a matched group of control subjects were inoculated with attenuated A/England/42/72 (MRC-7) virus. Evidence of infection was found in 14 of 23 (61 per cent) of control subjects and in seven of 23 (30 per cent) of immunized volunteers. This result showed a significant protection ($P = 0.04$) against challenge virus infection for volunteers given intranasal vaccine.

Introduction

Secretory antibody has been shown to have an important role in immunity to certain respiratory virus infections (Smith *et al.*, 1966; Perkins *et al.*, 1969), and a similar relationship has been suggested for influenza (Couch *et al.*, 1969; Waldman *et al.*, 1969). Since influenza vaccines in saline given intranasally selectively stimulated local production of antibody, this method of immunization has been used in a number of studies (Waldman *et al.*, 1968; Kasel *et al.*, 1969; Waldman *et al.*, 1970; Waldman and Coggins, 1972). However, local immunization with killed influenza vaccines did not stimulate serum antibody production as effectively as parenteral immunization (Gwaltney *et al.*, 1971; Waldman and Coggins, 1972; Shore *et al.*, 1974), and it was the titre of serum antibody which related most directly to immunity to influenza (Meiklejohn *et al.*, 1952; Hobson *et al.*, 1972). In addition, parenteral immunization with influenza vaccine has

been reported to induce local antibody as efficiently as intranasal immunization (Downie and Stuart-Harris, 1970; Rosenbaum *et al.*, 1973; Shore *et al.*, 1974).

Although the above results suggest that parenteral administration of vaccine is the preferred method of immunization against influenza, this conclusion is based on studies with intranasal saline vaccine, and cannot be extended to the freeze-dried, inactivated vaccine "Influvac"; this vaccine differs markedly both in form and method of application from other intranasal vaccines. The present study reports the serum and nasal wash antibody responses to Influvac in a group of volunteers. In addition, the vaccinees and a group of matched control volunteers were challenged with live homologous virus, and the incidence of infection in the two groups was compared.

Materials and Methods

Viruses

Influenza virus A/England/42/72 (H3N2) was kindly supplied by Dr. G. C. Schild, National Institute for Medical Research, Mill Hill, London. Virus pools were prepared by the allantoic inoculation of 10-day embryonated eggs. After incubation at 33° C for 48 hours, the allantoic fluids were collected, pooled and identified by HI tests using monospecific ferret anti-serum. The virus pool had a titre of $10^{8.3}$ EID_{50}/ml, and was stored at —80° C.

Virus Vaccine

Freeze-dried, inactivated A/England/42/72 vaccine (Influvac) was kindly given by Duphar Laboratories Ltd.; the inactivated vaccine virus was mixed with a Freon propellant in a pressurized container fitted with a valve adjusted to deliver a metered dose of 100 International Units (IU) of vaccine. Each volunteer was inoculated with two doses of vaccine into each nostril, giving a total dose of 400 IU. The vaccine had been shown to produce serum haemagglutination inhibiting antibody following intraperitoneal inoculation of mice (T. C. G. Smith, personal communication).

The MRC-7 virus (H3N2), a recombinant of influenza viruses 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; previous studies had shown this virus to be fully attenuated for man (A. S. Beare, personal communication). The virus was supplied in allantoic fluid, and was stored at —80° C. Immediately prior to inoculation into volunteers, the virus was thawed and diluted with phosphate buffered saline, pH 7.4 (PBS) to contain $10^{7.5}$ EID_{50}/1.0 ml; the diluted virus was maintained at 4° C, and was used within 30 minutes of preparation.

Experimental Design

A group of 50 medical students volunteered to take part in the study; all were healthy, with no history of allergy to eggs or to egg products. The volunteers were aged 20—26 years, and 18 were female. A specimen of blood was obtained from each volunteer, and titrated for haemagglutination inhibiting antibody to influenza virus A/England/42/72. From these results, the volunteers were divided into two matched groups; the volunteers in one group were to be immunized, and the second group served as controls.

Volunteers in the vaccine group were requested to blow their noses, and each subject was inoculated intranasally with 400 IU of inactivated A/England/42/72 vaccine through a disposable nose piece which fitted onto the vaccine container. The volunteers were asked not to blow their noses for an hour following immunization. Two nasal washings were collected from each vaccinee three and seven days prior to immunization and further specimens were collected weekly for three weeks following

immunization; these specimens were collected and processed by the methods described by DOWNIE and STUART-HARRIS (1970). A second blood sample was collected from each vaccinee three weeks after immunization.

Four weeks after intranasal immunization, the vaccinees, together with the matched group of control volunteers were each inoculated with MRC-7 virus. The subjects were asked to lie down on an examination couch with their heads fully extended backwards over the edge of the couch. 0.5 ml of diluted virus was inoculated into each nostril with a dropping pipette. Following inoculation, the volunteers remained lying for two minutes, and on rising were asked not to blow their noses for a further hour. A nasal washing and a throat swab were collected three days after virus infection for virus isolation studies, and a further blood sample was collected 18—21 days later.

Virus Isolation

Nasal washings, taken in PBS to which 2.0 per cent bovine serum albumin and antibiotics were later added, and throat swabs, taken into medium '199' containing 2 per cent foetal bovine serum and antibiotics, were stored at —80° C prior to testing for virus. The specimens were thawed, and 0.1 ml of undiluted specimen was inoculated allantoically into each of three or four 10-day embryonated eggs. After three days incubation at 33° C, the allantoic fluids were harvested, and tested for haemagglutination with fowl cells. All virus isolates were identified by HI tests, using specific ferret antisera.

Serological Tests

Haemagglutination-Inhibiting (HI) Tests

These tests were carried out by the microtitre method of SEVER (1962). Prior to testing, the serum specimens were treated with cholera filtrate (Burroughs Wellcome Ltd.) for 18 hours at 37° C and subsequently heated for 30 minutes at 56° C. Serum dilutions in PBS were mixed with an equal volume of virus containing eight (50 per cent) haemagglutinating units for 10—15 minutes before the further addition of an equal volume of 0.5 per cent fowl erythrocytes. The HI titres were read when the cells had settled at room temperature, and were expressed as the highest dilution of serum which caused 50 per cent reduction of virus haemagglutination.

Neutralization Tests

All nasal washings were titrated for neutralizing antibody to influenza virus A/England/42/72 by the allantois-on-shell (AOS) method (FAZEKAS DE ST. GROTH *et al.*, 1958). An 0.05 ml volume of virus containing $10^{3.0}$ EID_{50} was added to 0.5 ml of serial dilutions of nasal washings. After incubation for 30 minutes at 0° C, 0.05 ml of each virus-nasal wash mixture was inoculated on AOS fragments from 10-day embryonated eggs; each specimen was titrated on AOS pieces from four different eggs to allow for the variable sensitivity of eggs to virus infection. After incubation for 3 days at 35° C with constant shaking, the shell fragments were removed, and the culture fluids tested for virus by haemagglutination. The neutralizing antibody titre was calculated by the method of REED and MUENCH (1938).

IgA Determination

Nasal washings were concentrated 10-fold by dialysis against 30 per cent Carbowax, and the concentration of secretory IgA measured by the single radial diffusion method (MANCINI *et al.*, 1965). Human secretory IgA antiserum was obtained from Dakopatts, Copenhagen, Denmark, and a standard secretory IgA was prepared in this laboratory from human breast milk; purification of IgA was by block electrophoresis in Pevikon-Geon (FAHEY and MCLAUGHLIN, 1963), followed by DE.52 cellulose chromatography.

Protein Concentration

The protein concentration of nasal washings was determined by the method of LOWRY *et al.* (1951).

Results

Serum HI Antibody Titres of Volunteers

A serum specimen was obtained from each volunteer prior to immunization, and tested for HI antibody titre to influenza virus A/England/42/72. From these results the volunteers were divided into two groups; each volunteer in the group to be immunized had a corresponding control whose serum HI antibody titre was identical or very similar. If a volunteer failed to complete the experiment, he and the corresponding volunteer in the second group were eliminated from the study. Twenty three volunteers in each group completed the study, and a comparison of the serum HI antibody titres of these volunteers prior to immunization is shown in Table 1. In each group, 15 volunteers had serum HI antibody titre of $<1:20$, four had titres of 1:20—1:40 and four had antibody titres of $\geqslant 1:60$; the geometric mean titre (gmt) of serum HI antibody were almost identical for the two groups (Table 1).

Table 1. *Serum HI Antibody to Influenza Virus A/England/42/72 in Volunteers*

Group	No. tested	Serum HI antibody titre (A/Eng/72)	
		HI (Range)	HI (Mean; gmt)
Controls	15	<20	6.1
	4	20—40	29.1
	4	$\geqslant 60$	108.4
Vaccinees	15	<20	5.8
	4	20—40	29.1
	4	$\geqslant 60$	108.4

Serum and Local Antibody Response to Immunization

The serum HI antibody and the nasal wash neutralizing antibody responses of the vaccinees to intranasal immunization with 400 IU of A/England/42/72 vaccine is shown in Table 2. Of the thirteen subjects with serum HI antibody titres of $<1:10$, five developed low titres of antibody following immunization; however, the serum antibody response was poor, and for the 15 volunteers with serum HI antibody titres of $<1:20$, the gmt increased from 1:5.8 to 1:8.9 following immunization. Only one volunteer in this group showed a four-fold rise in serum HI antibody titre. For the four volunteers with pre-immunization serum HI antibody titres of 1:20—1:40, the gmt increased from 1:29.1 to 1:45.6, and for the volunteers with pre-immunization titres of $\geqslant 1:60$ the gmt increased from 1:108.1 to 1:138.6. Thus, even for volunteers with pre-existing serum HI antibody, immunization induced only small increases in serum antibody titre.

None of the volunteers produced local antibody prior to immunization; however, seven of the 15 volunteers with serum HI antibody titres of $<1:20$ prior to immunization developed nasal wash neutralizing antibody at 2—3 weeks following immunization (Table 2). In addition, six of eight volunteers with pre-immunization serum HI antibody titres of $\geqslant 1:20$ also produced detectable nasal wash antibody after immunization. The titres of nasal wash antibody were in all cases low. For the volunteers with no detectable serum HI antibody prior to immunization, the

gmt of local antibody increased from 1.0 to 1.6, and for the eight volunteers with pre-immune serum antibody, the titres of neutralizing antibody increased from 1.0 to 3.1.

Table 2. *Response of Volunteers to Intranasal Inoculation with Inactivated A/England/42/72 Vaccine*

Volunteer No.	Response to immunization			
	H.I. titre		N/W Neut. titre	
	Ac. Serum	Conv. Serum	pre N/W	post N/W
1	<10	<10	<2	<2
2	<10	15	<2	4
3	<10	20	<2	<2
4	<10	<10	<2	<2
5	<10	<10	<2	<2
6	<10	15	<2	3
7	<10	<10	<2	<2
8	<10	<10	<2	2
9	<10	<10	<2	4
10	<10	<10	<2	2
11	<10	15	<2	<2
12	<10	<10	<2	<2
13	<10	15	<2	4
14	15	15	<2	<2
15	15	30	<2	2
Mean (gmt)	5.8	8.9	1.0	1.6
16	30	60	<2	4
17	30	30	<2	6
18	40	80	<2	8
19	20	30	<2	<2
Mean (gmt)	20.1	45.6	1.0	3.2
20	80	80	<2	4
21	120	120	<2	<2
22	60	80	<2	6
23	240	480	<2	2
Mean (gmt)	108.4	138.6	1.0	2.6

Local Antibody Production and Nasal Wash Protein and IgA

In addition to the nasal washings taken two and three weeks after immunization for neutralizing antibody study, two specimens taken prior to and one specimen taken seven days after immunization, were also examined for protein and IgA concentration. No significant change in protein or IgA concentration of nasal washings was found following immunization, and since the concentration for all the specimens from a single volunteer was very similar, the protein and IgA levels in the five specimens from each volunteer were averaged. The relationship between neutralizing antibody titre, protein and IgA concentration is shown in Table 3. Antibody was detected in the nasal washings taken two and/or three weeks follow-

ing immunization from seven of the 15 volunteers with pre-immunization titres of serum HI antibody of $<1:20$, the remaining eight volunteers did not produce detectable levels of local neutralizing antibody (Table 3). The mean protein and IgA concentrations of nasal washings from the volunteers who developed antibody was greater than that of the eight volunteers who did not produce detectable nasal wash neutralizing antibody following immunization. In addition, although the serum HI antibody response to immunization was poor, volunteers who produced nasal wash antibody showed the better serum antibody response (Table 3). Only eight volunteers had serum HI antibody titres of $\geqslant 1:20$ to A/England/42/72 virus prior to immunization, and this was too few to allow a comparison of nasal antibody production with nasal wash protein and IgA levels in these vaccinees.

Table 3. *Antibody Response and Nasal Wash Protein and IgA Concentration in Volunteers Immunized Intranasally with Inactivated A/England/42/72 Vaccine*

Serum HI titre prior to immunization	Antibody response to immunization		Nasal wash determination	
	Nasal antibody	Serum HI response (gmt)	Protein (μgs/ml)	IgA (μgs/ml)
<20 (15)	+ve (7)	5.85—10.4	491 (257—708)	39 (26—47)
	—ve (8)	5.73—7.82	249 (77—350)	31 (23—49)
⩾20 (8)	+ve (6)	58.8—74.1	454 (147—635)	35 (23—58)
	—ve (2)	49.0—60.0	318 (167—469)	32 (23—41)

Response to Challenge Infection with MRC-7 Virus

Four weeks after immunization with A/England/42/72 the vaccinees, together with the matched group of control volunteers, were each inoculated with 1.0 ml of MRC-7 virus containing $10^{7.5}$ EID_{50}. The results are shown in Table 4. For the control volunteers, virus was recovered three days after challenge infection and a significant serum antibody response occurred in three subjects, only serological evidence of infection was obtained for a further 12 volunteers and virus was recovered from one volunteer who showed no serological rise in serum HI antibody titre. Thus, evidence of infection with MRC-7 virus was obtained for 14 of 23 (61 per cent) of the control group. In contrast, for the immunized group, virus was recovered from one volunteer who showed a significant serum antibody response, and serological evidence of infection was obtained for a further six subjects. Evidence of infection by MRC-7 virus was obtained in a total of seven of 23 (30.4 per cent) volunteers in the immunized group. This result showed that infection by the challenge virus occurred significantly less frequently in the immunized volunteers than in the control subjects ($P = 0.04$).

Of the seven immunized volunteers that were infected with challenge virus, two produced nasal wash antibody following immunization. In contrast, of the eight volunteers who were not infected with MRC-7 virus, five produced local antibody following immunization. Although a smaller number of infections were

observed in volunteers who developed local antibody production, the numbers are too small to give statistical significance. The lower number of infections in volunteers who produced local antibody may have been due to some other factor; thus, a serum antibody response was more commonly found in volunteers who produced local antibody, and the failure to infect these subjects may relate to this serum antibody response.

Table 4. *Results of Challenge Infection of Volunteers Previously Immunized with Intranasal A/England/42/72 Vaccine*

Group	Serum HI antibody titres[a]	No tested	Infection by MRC-7 virus			Total (%)
			Virus isolation	Significant HI antibody response	Total	
Vaccine	<1:20	15	1/15	7/15	7/15	7/23 (30.4)
	1:20—1:40	4	0/4	0/4	0/4	
	⩾1:60	4	0/4	0/4	0/4	
Control	<1:20	15	3/15	10/15	10/15	14/23 (61)
	1:20—1:40	4	0/4	2/4	2/4	
	⩾1:60	4	1/4	1/4	2/4	

[a] Serum HI titres prior to immunization.

Discussion

Inactivated influenza virus given intranasally in saline has been shown to induce local antibody (Waldman *et al.*, 1968; Kasel *et al.*, 1969; Waldman and Coggins, 1972; Downie and Stuart-Harris, 1970); however, this method of immunization induced a relatively poor serum antibody response (Shore *et al.*, 1974), and it is the serum HI antibody titre which most closely related to immunity to infection (Meikeljohn *et al.*, 1952; Hobson *et al.*, 1972). Since parenteral immunization induced relatively high titres of serum antibody and levels of nasal wash antibody comparable to those found following intranasal immunization (Potter *et al.*, 1973; Shore *et al.*, 1974), the evidence would indicate that parenteral inoculation of vaccine is the preferred method of immunization. These results should not be extended to "Influvac", since this product was physically distinct from saline vaccines; "Influvac" was a freeze-dried product which was administered with a Freon propellant to deliver a standard dose of a standard particle size (Duphar Laboratories, Ltd.).

Previous studies have shown that "Influvac" induced local antibody formation, and a poor serum HI antibody response (Liem *et al.*, 1973; Potter *et al.*, 1975); however, volunteers immunized with "Influvac" showed significant protection against natural influenza (Liem *et al.*, 1973; Haigh and Howell, 1973; Haigh *et al.*, 1973). In addition, immunization with "Influvac" containing inactivated A/Aichi/68 (H3N2) virus gave significant protection against natural influenza due to A/England/42/72 (H3N2) virus (Haigh and Howell, 1973). Although the conclusions of the above studies were tested by statistical analysis of the results, the influenza attack rate among the groups studied was low. In a small group study, volunteers immunized with "Influvac" containing A/Hong

Kong/68 were relatively more resistant to infection by attenuated A/England/42/72 virus than non-immunized; however, in this study the numbers of volunteers was too small for statistical analysis (Potter *et al.*, 1975).

In the present study, immunization with "Influvac" induced a poor serum antibody response, but 13 of the 23 volunteers developed detectable nasal wash antibody; this result was similar to that of a previous study (Potter *et al.*, 1975). The production of nasal wash antibody was more commonly found in volunteers who produced some increase in serum antibody; this result was similar to the findings for parenteral immunization, where local antibody production was directly related to the serum antibody response (Wenzel *et al.*, 1973). The production of nasal wash antibody was also related to the concentration of protein and IgA present in the nasal washing; thus, nasal wash neutralizing antibody was most frequently detected in washings containing relatively high concentrations of protein and IgA. Whether this was because washings with high protein levels were more likely to be efficiently taken and, therefore, more likely to contain antibody, or that washings contained relatively high titres of protein were from volunteers with more reactive nasal mucosa and therefore from persons more likely to respond to vaccine is not known.

The results of challenge infection of volunteers previously immunized with "Influvac" showed that seven of 23 vaccinees were infected with the MRC-7 virus; in contrast, 14 of 23 control volunteers were infected with challenge virus. This result indicated a significant level of protection in the immunized group. It can be argued that better protection could have been achieved by parenteral immunization, and in this respect the present vaccine may suffer by comparison with vaccines given by injection. However, the intranasal vaccine produced no reaction in the present study; in contrast, parenteral immunization has been reported to cause local and occasional systemic reaction, particularly in children (Quilligan *et al.*, 1949; Davenport *et al.*, 1964). In comparisons of intranasal and parenteral vaccines, the former were reported to produce significantly less reaction (Waldman *et al.*, 1969; Waldman and Coggins, 1972); in addition, an intranasal vaccine may be more acceptable to the public than parenteral immunization, and for this reason the potential of this method of immunization should be further explored.

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