

**The sequential appearance of antibody
and immunoglobins in nasal secretion after immunization
of volunteers with live and inactivated
influenza B virus vaccines**

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SUMMARY

The sequential development of the immune response in nasal washings was studied in 54 volunteers immunized with either attenuated or inactivated influenza B/Eng/13/65 virus vaccines.

Eleven of the 15 volunteers given the inactivated vaccine by deep subcutaneous inoculation showed no rise in nasal wash protein or immunoglobins due to the immunization procedure nor was specific neutralizing antibody detected in their nasal washings after immunization. Neutralizing antibody was detected in nasal washings of three volunteers in this group who also showed a 20-fold or greater increase in serum haemagglutinin-inhibiting antibody after immunization and in one volunteer who had antibody present in pre-trial nasal washings.

Eleven of 15 volunteers who were successfully infected by the live attenuated vaccine showed a characteristic rise in protein and IgA and IgG immunoglobulin concentrations in nasal washings 5–14 days after the administration of the live virus vaccine. Neutralizing antibody was detected in the nasal washings of these 11 volunteers and appeared at the same time as or 1–2 days after the initial rise of protein and immunoglobulin. Neutralizing antibody was also detected in the nasal washings of one other volunteer who did not show a rise in protein or immunoglobulin concentration in nasal washings after immunization.

IgA was detected (≥ 3 mg./100 ml.) in the majority (84%) of nasal wash specimens which had a protein concentration of 0.2 mg./ml. or greater while IgG was not detected (≥ 4.5 mg./100 ml.) until the protein concentration rose to 0.4 mg./ml. or greater. The geometric mean concentration for normal nasal wash protein in this study was 0.3 ± 0.1 mg./ml.

Regression analysis indicated that the concentrations of both IgA and IgG immunoglobins were directly proportional to the protein concentration in nasal washings but that this relationship varied considerably between individuals.

Absorption studies indicated that neutralizing and haemagglutinin-inhibiting antibodies in nasal secretion to influenza B/Eng/13/65 virus were predominantly associated with the IgA class of immunoglobulin.

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INTRODUCTION

Early studies (Burnet, Lush & Jackson, 1939; Francis, 1941; Fazekas de St Groth & Donelley, 1950) indicated that neutralizing antibody to influenza virus in respiratory secretions might play an important part in resistance to further infection.

Artenstein, Bellanti & Buescher (1964), Remington, Vosti, Lietze & Zimmerman (1964), Rossen, Schade, Butler & Kasel (1966) and Alford, Rossen, Butler & Kasel (1967), have since confirmed that neutralizing activity found in nasal secretion after respiratory virus infections was due to specific antibody and belonged to a different immunoglobulin class (IgA) than that found in serum (IgG).

IgA (11S) has been found to be the predominant immunoglobulin in nasal secretion and is generally thought to be synthesized locally (Tomasi, Tan, Solomon & Prendergast, 1965; South *et al.* 1966; Butler, Rossen & Waldman, 1967; Rossen *et al.* 1968). However, the presence of IgG in nasal secretion has not been well documented and until recently (Butler *et al.* 1970) there has been little published information on the sequential appearance of these two immunoglobulins in relation to nasal secretion antibody after infection or immunization by respiratory viruses.

The present study investigated the sequential development of the immune response in nasal secretion with particular attention to the production of IgG and IgA immunoglobulins after three immunization procedures using live and inactivated influenza B virus vaccines.

MATERIALS AND METHODS

Virus vaccines

A live attenuated influenza B/Eng/13/65 virus vaccine (Beare, Bynoe & Tyrrell, 1968) was kindly supplied by Dr D. C. Breeze, Evans Medical Ltd., Speke. This virus had a titre of $10^{9.4}$ EID₅₀/ml. Formalin-inactivated vaccines containing 5.5×10^8 haemagglutinating units (HAU)/ml. were prepared by Evans Medical Ltd. from an influenza A/Eng/1/61 (H2N2) virus strain and from the same strain of influenza B virus as the live attenuated vaccine.

Volunteers

The design of the vaccine trials has been described previously (Downie & Stuart-Harris, 1970). A total of 54 medical students were immunized with influenza B virus vaccines during two vaccine trials in October 1967 and October 1968. In the 1967 trial, eight volunteers were given an intranasal dose of 1.0 ml. of the live attenuated influenza B virus vaccine diluted in Hanks's saline to contain $10^{5.4}$ EID₅₀/ml. of virus and a second group of seven volunteers was inoculated by deep subcutaneous injection with 1.0 ml. of the inactivated influenza B virus vaccine. In the 1968 trial 11 students were given 1.0 ml. of either the live influenza B virus vaccine ($10^{5.4}$ EID₅₀/ml.) or 1.0 ml. of the inactivated influenza B virus vaccine intranasally, a further eight were given 1.0 ml. of the same inactivated vaccine subcutaneously and a control group of nine students was given 1.0 ml. of the influenza A2/Eng/1/61 virus vaccine subcutaneously. One month after the

initial immunization, all the volunteers were given an intranasal dose of 1.0 ml. of live influenza B/Eng/13/65 virus vaccine diluted to contain $10^{6.4}$ EID₅₀/ml. of virus. The first inoculation of virus in the 1968 vaccine trial was designated as the immunizing dose and the second as the challenge dose of virus.

Serial nasal washings were collected as described previously (Downie & Stuart-Harris, 1970) before and after immunization and after challenge during a total period of 21 days in 1967 and 53 days in 1968. The nasal washings were tested for the presence of haemoglobin by the guaiac test and positive specimens were discarded. Serum specimens were collected before immunization, 3 weeks after immunization and 3 weeks after challenge and stored at -20° C.

Haemagglutination-inhibition (HI) test

Assays of haemagglutinin (HA) and the inhibition of haemagglutination (HI) by serum and nasal washings were done according to the method described by Fazekas de St Groth & Webster (1966). Before testing, all nasal washings were treated with receptor-destroying enzyme (RDE). A normal rabbit serum, a normal nasal washing containing 2 mg./ml. of protein and a specific rabbit antiserum of known titre to influenza B/Eng/13/65 virus were included as controls.

Neuraminidase-inhibition (NI) test

Assay of neuraminidase-inhibiting (NI) antibody was done as described previously (Downie, 1970).

Neutralization test

Titration of neutralizing antibody in nasal washings were done according to the Quantitative Haemadsorption (QH) test described by Finter (1967). A specific rabbit antiserum of known titre was included in each test and the nasal washings were heated at 56° C. for 30 min. before testing.

Complement fixation (CF) test

CF tests were carried out by standard methods using two exact units of complement, and overnight fixation at 4° C.

Assay of immunoglobins

The immunoglobins in nasal washings were assayed by the radial diffusion precipitation method in agar (Mancini, Carbonava & Heremans, 1965). A stabilized human reference serum (Hoechst Pharmaceuticals Ltd., London) was used to measure IgA, IgG and IgM immunoglobulin levels in nasal washings. Since the rate of diffusion in agar is dependent upon molecular size, the values obtained for nasal washing IgA (11S) will be falsely low in comparison to serum IgA (7S) and therefore will not be absolute values for this immunoglobulin. However, comparison between different nasal wash specimens will still be valid.

IgA and IgG were specifically absorbed from nasal washings with an equal volume of anti-human immunoglobulin serum at optimal precipitating concentrations as previously described (Downie, 1970). Goat anti-human IgA serum was

obtained from Hyland Laboratories, Los Angeles, California, and goat anti-human IgG and IgM sera were kindly supplied by Professor P. G. H. Gell, Department of Experimental Pathology, Birmingham University, England.

Protein assay

Protein concentrations of nasal washings were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard.

RESULTS

Relation of protein and immunoglobins in nasal washings

Protein estimations were done on every nasal washing collected from all volunteers during 1967 and 1968. The observations for 1967 and 1968 showed that the geometric mean concentration for normal nasal wash protein for the volunteers was 0.3 ± 0.1 mg./ml. and concentrations of greater than 1.0 mg./ml. were considered to be abnormal.

Quantitative assay for IgA, IgG and IgM were done on every nasal washing collected from all volunteers during 1967 and from those volunteers in 1968 who showed a fourfold rise in serum antibody after immunization or challenge or who showed a threefold rise in nasal wash protein concentration. IgM was detected in 14 of the 1100 nasal wash specimens assayed for immunoglobins. All 14 specimens were collected during a rise in protein either due to the live influenza B virus vaccine or an unrelated common cold-like infection and the mean concentration of protein in these specimens was 5.2 mg./ml.

IgA was generally found in measurable amounts (≥ 3 mg./100 ml.) when the concentration of nasal wash protein was 0.2 mg./ml. or greater, and IgG was generally measurable (≥ 4.5 mg./100 ml.) when the protein concentration was 0.4 mg./ml. or greater. Since the protein concentration of normal wash was 0.3 mg./ml., the majority (84 %) of specimens contained measurable concentrations of IgA (3–6 mg./100 ml.) but not IgG.

The relation between protein concentration and the concentrations of IgA and IgG in nasal washings was examined using grouped data regression analysis on results obtained from 27 individuals totalling 347 observations for IgA and 248 for IgG (Figs. 1, 2). A group consisted of all observations on IgA or IgG at a particular concentration of protein. A linear relationship best fitted to the values for both immunoglobins was calculated by the method of least squares (Dixon & Massey, 1957) and the slopes of these lines were significantly different from zero ($P < 0.01$). This suggested that both nasal secretion IgA and IgG were proportional to the concentration of nasal secretion protein. Thus, at protein concentrations of 1.0 mg./ml. or greater both IgA and IgG tended to have values of 10 mg./100 ml. or greater. However, both analyses showed significantly higher deviations from the line of regression than would be expected on the basis of observed variation within groups although the contribution of regression to variation in both IgA and IgG was still highly significant. Thus, the relation could not be regarded as a simple one. One possibility was that it was curvilinear, although similar analyses

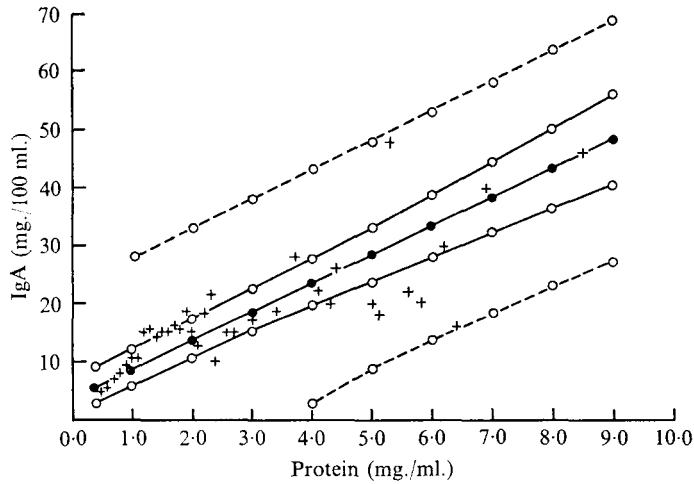


Fig. 1. Grouped data regression equation analysis of IgA immunoglobulin concentration and nasal wash protein concentration (347 observations). ●—●, Line of best fit; ○—○, 90% limits (fiducial) on predicted value; ○--○, tolerance limits for predicting future single values.

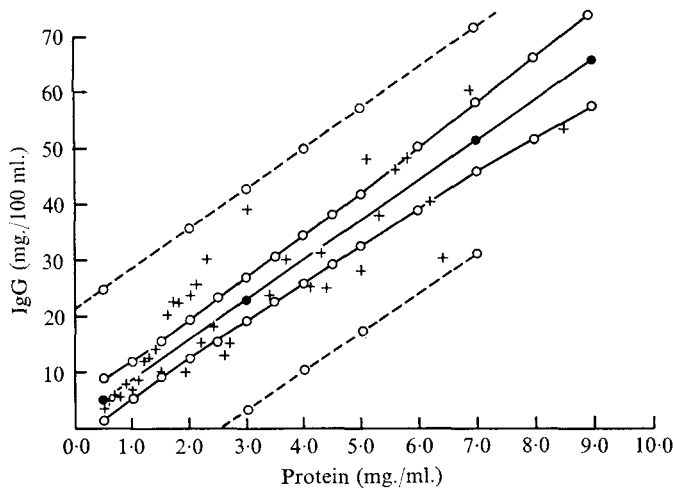


Fig. 2. Grouped data regression equation analysis of IgG immunoglobulin concentration and nasal wash protein concentration (248 observations). ●—●, Line of best fit; ○—○, 90% limits (fiducial) on predicted value; ○--○, tolerance limits for predicting future single values.

using $X = \log(10 \times \text{protein})$ still showed significant deviations from regression between groups. Another possibility was that different individuals were making a greater contribution to the variance between groups than to the within-group variance, i.e. that the relation of protein concentration to IgA and IgG concentrations varied from individual to individual. To test this possibility the same analysis was carried out separately on four individuals and it was found that the majority of the deviations from regression due to the variance between groups almost entirely disappeared. Thus, the relation between IgA and IgG and protein con-

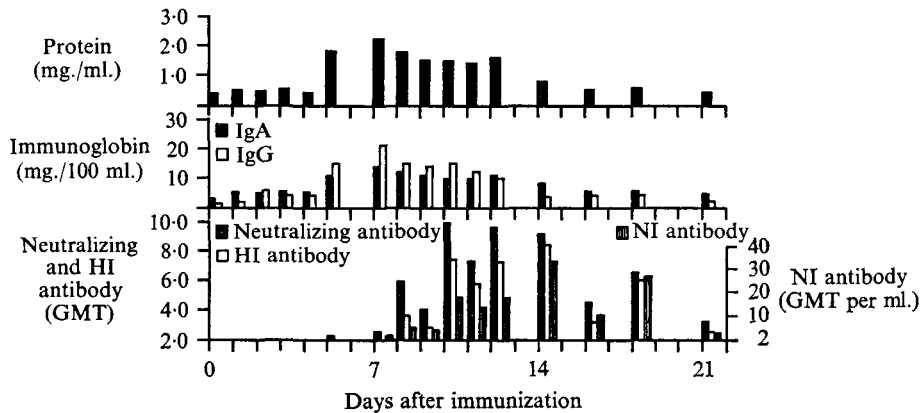


Fig. 3. Sequential changes in protein, immunoglobulin and antibody concentrations in the nasal washings of 15 volunteers showing serological evidence of infection after either the immunization or challenge dose of live attenuated influenza B virus vaccine. Neutralizing HI and NI antibody are expressed as the reciprocal of the geometric mean titres (GMT). Protein and immunoglobulin concentrations are expressed as the arithmetic mean.

centrations was highly significant ($> 0.1\%$) for each individual. Joint regression analyses were performed to test the hypothesis of individual difference and the best fitting lines describing the relation of protein concentration with the concentration of IgA and IgG differed significantly in slope between individuals. Thus, much of the variation between groups due to deviations from regression must have stemmed from these individual differences.

Immune response in nasal washings after immunization

The sequential development of the immune response in nasal secretion to immunization with live and inactivated influenza B/Eng/13/65 virus vaccines was studied by assaying serial nasal washings from each volunteer for protein, immunoglobulins (IgA, IgG and IgM), and specific virus neutralizing, NI, and HI antibodies. Antibody titres were adjusted to values per ml. and expressed as the reciprocal of the geometric mean titres (GMT). Protein and immunoglobulin concentrations were expressed as the arithmetic mean.

Immune response in nasal washings - live virus vaccine given intranasally

Neutralizing, HI and NI antibodies were detected in the nasal washings of 12 out of 15 volunteers who showed serological evidence of infection after either the immunization or challenge dose of live influenza B virus vaccine. These 15 volunteers included 6 from the 1967 trial and 1 from the 1968 trial who were immunized with the live virus vaccine, and 8 from the 1968 trial who were challenged with the live virus vaccine.

Eleven of the 12 volunteers with nasal-wash antibody also showed a characteristic increase in the concentration of nasal wash protein and immunoglobulins to levels of from three to five times greater than that found before immunization. The sequential changes in nasal-wash protein, immunoglobulins and antibody con-

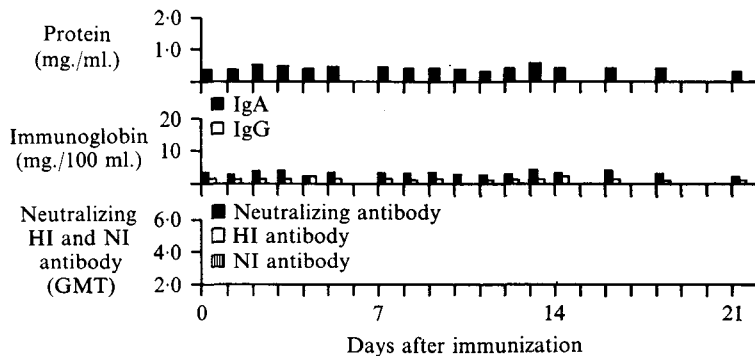


Fig. 4. Sequential changes in protein, immunoglobulin and antibody concentrations in the nasal washings of 11 volunteers successfully immunized with inactivated influenza B virus vaccine by deep subcutaneous injection. Neutralizing, HI and NI antibody are expressed as the reciprocal of the geometric mean (GMT). Protein and immunoglobulin concentrations are expressed as the arithmetic mean.

centrations of the 15 volunteers who responded serologically to the live virus vaccine are summarized in Fig. 3.

The rise in nasal-wash protein concentration (> 1.0 mg./ml.) and IgA and IgG immunoglobulin concentrations (> 10 mg./100 ml.) was observed 5-14 days after the administration of live virus and lasted from 1 to 7 days in different individuals. IgG was generally detected only at the time of this increase in protein and reached maximum concentrations of 10-60 mg./100 ml. with a maximum mean of 21.6 mg./100 ml. while IgA concentrations (7S standard) ranged from 10 to 40 mg./100 ml. with a maximum mean of 15.4 mg./100 ml. Fourteen to 21 days after immunization the protein and immunoglobulin concentrations returned to their normal values.

Neutralizing, HI and NI antibody appeared in the nasal washings 7-14 days after the administration of the live virus vaccine and could be detected up to 28 days.

Immune response in nasal washings - inactivated virus vaccine given subcutaneously

Eleven of the 15 volunteers given the inactivated influenza B/Eng/13/65 virus vaccine subcutaneously in the 1967 and 1968 trials showed no abnormal rise in nasal wash protein (> 1.0 mg./ml.) or immunoglobins (> 10 mg./ml.) due to the administration of vaccine, nor was neutralizing antibody to influenza B/Eng/13/65 virus detected in their nasal washings during the 21- to 28-day period after immunization. These results are summarized in Fig. 4 and are in sharp contrast to those shown in Fig. 3.

However, neutralizing antibody to influenza B/Eng/13/65 virus was detected in the nasal washings of the four remaining volunteers in this group; three showed a 20-fold or greater rise in serum HI antibody to the inactivated vaccine while the fourth had neutralizing antibody present in nasal washings before the administration of the vaccine.

Fig. 5 illustrates the pooled results for changes in nasal wash protein, IgA, IgG, and antibody concentrations of the three volunteers who had a 20-fold rise in serum HI antibody. Two of them were among six volunteers in the 1968 trial who

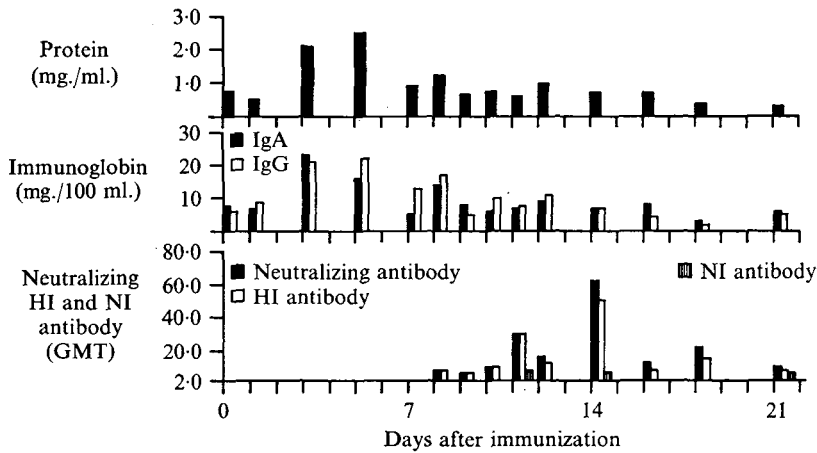


Fig. 5. Sequential changes in protein, immunoglobulin and antibody concentrations in the nasal washings of three volunteers showing a 20-fold or greater rise in serum HI antibody after immunization with inactivated influenza B virus vaccine by deep subcutaneous injection. Neutralizing HI and NI antibody are expressed as the reciprocal of the geometric mean titres (GMT). Protein and immunoglobulin concentrations are expressed as the arithmetic mean.

had a common cold-like illness unrelated to the immunization procedure during the post-immunization period which caused an increase in protein and immunoglobulins 1–5 days after administration of the vaccine. In these two volunteers, nasal-wash protein, IgA and IgG concentrations were consistently higher than average (> 0.5 mg./ml., and > 6 mg./100 ml., and > 5 mg./100 ml. respectively) throughout the trial period. Influenza B/65 virus neutralizing and HI antibodies were detected in their nasal washings in relatively high titres (GMT 1/30–1/60) from the 11th day after immunization to the end of the period of observation. NI antibody on the other hand was detected on only three occasions and in low concentration (GMT 1/7), although the serum titres for NI antibody were comparable with the titres in those who were infected by the live virus vaccine.

*Immune response in nasal washings – inactivated virus
vaccine given intranasally*

Only one out of the 11 volunteers in the 1968 vaccine trial given inactivated influenza B virus vaccine intranasally responded serologically to the immunization procedure. There was no abnormal rise in nasal-wash proteins or immunoglobulins due to the immunization procedure. Neutralizing antibody to influenza B/65 virus was detected in one specimen taken 8 days after immunization.

Relation of immunoglobulins and antibody in nasal washings

IgA and IgG immunoglobulins were specifically removed from nasal-wash specimens of volunteers immunized and challenged with influenza B/Eng/13/65 virus by precipitation with an equal volume of anti-human immunoglobulin sera at optimal proportions. The absorbed washings were then tested for the presence of

Table 1. Presence of neutralizing and HI antibody after specific absorption of IgA and IgG from nasal secretion of volunteers immunized and challenged with influenza B/Eng/13/65 virus (1968)

Volunteer and immunization procedure	Day after immunization*	Neutralizing antibody titre after absorption†				HI antibody titre after absorption†			
		Control	IgA absorbed	IgG absorbed	IgA-IgG absorbed	Control	IgA absorbed	IgG absorbed	IgA-IgG absorbed
Volunteer no. 6.	40	40	0‡	40	0	64	64	64	0
Live influenza B/Eng/13/65 intranasal	46	10	0	5	0	32	16	16	0
Volunteer no. 31.	11	40	0	5	0	64	64	64	0
Inactivated influenza B/Eng/13/65 (subcutaneous)	12	80	0	20	0	64	64	64	0
Volunteer no. 35.	31	40	0	10	0	64	64	64	0
Inactivated influenza A2/Eng/1/61 (subcutaneous)	44	30	0	20	0	64	32	32	0
	42	10	0	5	0	16	16	16	0

* Volunteers were challenged on day 28.

† Neutralizing antibody titre < 5.

‡ Reciprocal of titre.

§ HI antibody titre < 8.

neutralizing antibody and, after treatment with RDE, for HI antibody. IgM immunoglobulin was not detected in any of the specimens absorbed.

Table 1 gives the results from three volunteers in the 1968 trial for neutralizing and HI antibody in nasal washings after the absorption of IgA and IgG. In each instance removal of IgA immunoglobulin also removed the neutralizing activity of the nasal washing, while removal of IgG had little or no effect on this activity. Absorption of IgA also removed the HI antibody from nasal secretions in all but one instance. This nasal washing was taken from volunteer no. 6, 12 days after the administration of the challenge dose of virus. Absorption of either IgA or IgG had no effect on the HI titre to influenza B virus, suggesting that specific antibody was associated with both immunoglobins. Removal of both immunoglobins also removed the HI antibody activity. A later specimen from the same volunteer, taken 18 days after challenge, showed that the HI activity was entirely in the IgA fraction. One possible explanation might be the transudation of serum IgG antibody into respiratory tract secretions. Therefore serum and nasal washings from volunteers no. 6, 31, 35 and three others who showed HI antibody rises in both serum and nasal washing to either immunization or challenge with influenza B/Eng/13/65 virus in 1968, were treated with RDE and tested for HI antibody to influenza A/Tokyo/3/66 (H2N2) virus. If transudation of serum antibody into nasal washings was primarily involved one might expect HI antibody to the A/Tokyo/3/66 virus in both serum and nasal washings.

All six volunteers had serum HI antibody titres greater than 1/24 to influenza A/Tokyo/3/66 virus but none of the nasal washings from the six had detectable amounts of HI antibody to this influenza A virus. These results suggest that transudation of antibody from the serum to the nasal secretion was probably not the primary source of antibody in the nasal washings.

DISCUSSION

The results of the present study have indicated that there were considerable differences in the sequential development of the immune response in nasal washings of the volunteers who received either live or inactivated influenza vaccines. The live influenza virus vaccine given intranasally was found to be more successful in stimulating neutralizing, HI and NI antibody in nasal washings than was the inactivated vaccine given either intranasally or subcutaneously.

In this study a characteristic rise in nasal-wash protein occurred 5–14 days after the administration of live virus and coincided with similar increases in the concentration of IgA and IgG immunoglobins. Neutralizing and HI antibodies reached maximum titres after 10–14 days and were generally still detected up to 28 days after the administration of the live virus vaccine when protein and immunoglobulin concentrations had returned to pre-immunization levels.

These results are in agreement with previous studies (Rossen *et al.* 1970; Alford *et al.* 1967) in which neutralizing antibody appeared in nasal secretion between 7 and 28 days after administration of live influenza A virus, while the rise in nasal wash protein occurred 4–6 days after inoculation.

In contrast, inactivated influenza B virus vaccine given subcutaneously was

more efficient in stimulating serum antibody than antibody in nasal washings. The only volunteers in this group who developed detectable neutralizing antibody in nasal secretion after immunization also had a 20-fold or greater rise in serum HI antibody. Thus it seems probable that very high levels of circulating antibody need to be stimulated by subcutaneous vaccine before neutralizing or HI antibody can be detected in nasal secretion (Mostow *et al.* 1970). These results are consistent with the findings of other studies that commercially available inactivated vaccines given subcutaneously are poor inducers of nasal-secretion neutralizing antibody (Smith, Purcell, Bellanti & Chanock, 1966; Mann *et al.* 1968; Waldman, Mann & Kasel, 1968; Kasel *et al.* 1969; Fluk *et al.* 1970).

In the present study, considerable concentrations of IgG were found in nasal washings after the administration of live influenza B virus vaccine while in other studies (Rossen *et al.* 1965, 1966; Alford *et al.* 1967) only minimal amounts of IgG have been detected in nasal washings after influenza A virus infection. Moreover, in the present study, regression analysis indicated that the concentrations of both IgA and IgG were related to the concentration of both IgA and IgG were related to the concentration of protein in nasal wash and a rise in protein generally coincided with an increase in the concentration of both these immunoglobins. The relation between IgA and IgG and nasal wash protein was found to be highly significant when individuals were analysed separately (< 0.1%) but joint regression analysis indicated that this relation varied considerably between individuals.

Other findings suggest that there may be differences in the pattern of the local response in the host to different respiratory viruses. Butler *et al.* (1970) have shown that after infection by either rhinovirus type 15 or coxsackievirus A type 21, rises in the concentrations of IgA and nasal wash protein appeared to coincide while changes in IgG concentration appeared to follow those in nasal wash albumin. The peak concentration for IgG and albumin occurred 2–5 days earlier than that for IgA and nasal wash protein.

Specific absorption tests in this and in a previous report (Downie, 1970) have indicated that the neutralizing, HI and neuraminidase-inhibiting (NI) antibody activities in nasal secretion were predominantly associated with the IgA class of immunoglobulin. These results confirm and extend those of other workers (Rossen *et al.* 1966; Alford *et al.* 1967; Mann *et al.* 1968) who have demonstrated that the neutralizing antibody to influenza virus in nasal secretion was predominantly associated with the 11 S IgA immunoglobulin fraction.

Although substantial amounts of IgG were detected in nasal washing after the administration of live influenza B virus vaccine, it appeared to have little or no specific antibody activity. In only one instance in the present study did HI antibody to influenza B virus appear to be partly associated with IgG as well as with IgA immunoglobulin. The role that this nasal-wash IgG may play in the immune response to respiratory infection is not known but earlier studies (Artenstein *et al.* 1964; Bellanti, Artenstein & Buescher, 1965; Alford *et al.*, 1967) have indicated that part of the neutralizing antibody to influenza A virus in nasal secretion may be associated with 7S IgG immunoglobulin.

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