The production of neutralizing activity in serum and nasal secretion following immunization with influenza B virus

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SUMMARY

Trials were made in volunteers in 1967 and 1968 of various virus vaccines against influenza virus B. Sera and serially collected nasal washings before and after immunization were tested respectively for haemagglutination-inhibiting and tissue culture virus-neutralizing antibodies to the same strain of influenza B/Eng/65 virus as that used in the vaccines. Infection, as determined by recovery of virus and serological changes following intranasal instillation of attenuated live virus, was accompanied by the subsequent appearance of neutralizing antibodies in nasal secretion. Inactivated vaccine subcutaneously did not evoke nasal antibody formation in 1967 but did so in 1968.

In 1968 intranasal challenge of the volunteers with the attenuated virus 1 month after immunization demonstrated a correlation of susceptibility or resistance to infection with nasal and serum antibodies. Resistance appeared to depend either on a high level of serum antibodies or nasal antibodies, or both.

INTRODUCTION

In contrast to many other virus infections of man, the protection resulting from a single attack of influenza virus infection in the adult has long been believed to be temporary. Nevertheless remarkably few data exist concerning the duration of immunity following proven influenza virus infection. Though Sigel, Kitts, Light & Henle (1950) reported recurrent infections in 1949 among schoolboys affected in 1947 by the same influenza virus A1, such a brief duration of immunity seems exceptional. J. Fry (personal communication) indeed believes from observations in his own practice that relatively few adults affected by Asian influenza in 1957 were again affected clinically during the recurrent outbreaks of 1959, 1961 and 1964.

Because of the difficulty of judging influenzal immunity after natural infections, the challenging effect of deliberate inoculation with living virus has been studied in volunteers. Thus more than 20 years ago laboratory-cultivated influenza viruses A and B were used to challenge the protection induced by subcutaneous immunization (Henle, Henle & Stokes, 1943; Francis, Salk, Pearson & Brown, 1945; Salk, Pearson, Brown & Francis, 1945).

More recently viruses modified by artificial cultivation in hens' eggs or tissue cultures and attenuated in virulence for man have been used as vaccines and also

to test immunity (McDonald, Zuckerman, Beare & Tyrrell, 1962; Beare *et al.* 1967; Beare, Bynoe & Tyrrell, 1968; Beare *et al.* 1969). As these viruses provoke little or no clinical reaction, resistance to their infection has been assessed by attempted reisolation of virus from the throat and antibody responses in the serum. McDonald *et al.* (1962), using the attenuated IKSHA A2 virus in volunteers, showed that intranasal inoculation of living virus was less likely to result in infection in persons whose sera contained demonstrable neutralizing antibodies than in those without such antibodies. Nevertheless, these same experiments showed a lack of precise correlation between serum antibody titres and resistance to challenge infection such as was observed 30 years ago in relation to clinical influenza in the field (Hoyle & Fairbrother, 1937; Stuart-Harris, Andrewes & Smith, 1938; Rickard, Horsfall, Hirst & Lennette, 1941). Some persons with moderate titres of serum antibody were infected and conversely some persons without such antibodies resisted infection (Andrews *et al.* 1966; Beare *et al.* 1967).

Nasal antibody inhibitory to influenza virus A was first demonstrated by Francis (1940) and was found to increase after infection by Francis & Brightman (1941). Its increase following subcutaneous influenzal vaccination was suggested by Francis, Pearson, Sullivan & Brown (1943) and Mulder, Brans & Hers (1952). However this early work involved the collection of nasal secretion by a method which may have led to transudation of plasma or blood and immunological tests on nasal secretion require that secretion should be collected by simple wash-out with saline. By this method Alford, Rossen, Butler & Kasel (1967), and Mann *et al.* (1968) studied nasal secretions from volunteers either infected with living A2 virus or inoculated subcutaneously with inactivated virus vaccine. Little neutralizing antibody was found in the nasal secretion before these procedures but afterwards antibody titres rose in both groups, though to a much greater extent in those receiving live virus than in those inoculated with vaccine.

Waldman *et al.* (1968) used the respiratory route to introduce inactivated virus into the body by means of a coarse intranasal spray. Serum antibody responses were less than after subcutaneous inoculation but increases were found in the nasal antibody titre more often after intranasal application of vaccine. Kasel *et al.* (1969) have confirmed that a better nasal antibody response occurs after intranasal than after subcutaneous inactivated polyvalent influenza vaccine.

The present study was designed to compare the changes in the nasal secretions of volunteers following immunization with various influenza B vaccines and to attempt to correlate antibodies in the serum or nasal secretion with resistance to a test infection.

METHODS

Virus vaccines

Influenza B/Eng/13/65 virus (Beare *et al.* 1968), which had been passaged six times in leucosis-free eggs, was kindly supplied to Dr D. C. Breeze, Evans Medical Limited, Speke. This virus had a titre of $10^{9.4}$ egg infective doses (EID 50) per ml. It was used intranasally in a volume of 1.0 ml. (0.5 ml. into each nostril) diluted in balanced Hanks's saline to contain $10^{5.4}$ EID 50 per ml.

Formalin-killed vaccines were prepared from the same virus B strain or an A2 virus (A2/Eng/1/61) by Evans Medical Limited, and were given by deep subcutaneous inoculation in 1.0 ml. doses containing 5500 haemagglutinating units.

Nasal specimens

Nasal washings were collected by the volunteers themselves before and during the period of observation of each trial. Specimens were obtained by instilling approximately 2 ml. of sterile phosphate buffered saline (pH 7.2) into each nostril of the subject with the head held well back and the glottis closed. The fluid was then forcibly expelled into a sterile Petri dish. This procedure was repeated four or five times. In practice, the average specimen obtained was 9 ml. of nasal secretion. The washings were shaken vigorously with glass beads, centrifuged at 3000 rev./min. for 15 min. and the supernatant fluid stored at -20°C . The specimens were tested for the presence of haemoglobin by the guaiac test and positive specimens were discarded.

Serological methods

Tissue culture haemadsorption test. The nasal secretions were first heated at 56°C . for 30 min. In the test undiluted secretion was mixed with an equal volume of 10 or 100 tissue culture infective doses of influenza virus B (TCID₅₀) as determined by haemadsorption and held at 4°C . for 1 hr. Each of four tubes of Patas monkey kidney cultures was inoculated with 0.2 ml. of the mixture, incubated for 3 days at $35-36^{\circ}\text{C}$. and examined for haemadsorption with 0.5 % guinea-pig cells. Antibody was considered to have been present if there was more than a 75 % reduction in haemadsorption in comparison with control cultures of virus only.

Haemagglutination inhibition (H.I.) test. Serum specimens were diluted 1/6 with cholera filtrate (Philips Duphar, Holland) and incubated overnight at 37°C . Enzyme was then inactivated by incubation at 56°C . for 60 min. before testing. H.I. tests were carried out in WHO plastic trays using 0.2 ml. volumes of serum and an equal volume containing eight haemagglutinating units of virus. The virus-serum mixtures were incubated at room temperature for 60 min. before adding 0.2 ml. of 0.5 % fowl red blood cells.

Complement fixation (C.F.) test. The sera were tested for complement-fixing antibodies by standard methods using 2 MHD of complement and overnight fixation at 4°C . Before testing they were inactivated at 56°C . for 30 min. (Bradstreet & Taylor, 1962). The antigen used in the complement fixation test was kindly supplied by Dr C. M. P. Bradstreet, Public Health Laboratory Service, Colindale.

Virus isolation

Throat swabs were taken from volunteers given the live vaccine and from all the volunteers after challenge on the 2nd and 3rd day after inoculation. The swabs were placed in transport medium containing 2.5 % bovine serum albumin and polymyxin 125 units/ml. and stored at -70°C . Specimens for virus isolation were inoculated into the amniotic sac of 10-day-old embryonated hens' eggs, and into

Rhesus monkey kidney tissue culture cells. Eggs were harvested after 3 days incubation at 33°C. and the amniotic fluids were tested for virus haemagglutinins. Monkey kidney cells were tested for haemadsorption after 3–5 days incubation. Negative specimens were passed three times in eggs and twice in tissue culture before being discarded.

Tissue cell cultures

Patas monkey kidney cells were obtained from Burroughs Wellcome Limited, Beckenham, Kent, and Rhesus monkey kidney cells were supplied by the Biological Standards Division of the National Institute for Medical Research. These cells were grown in mixture '199' fluid with 5 % calf serum at an initial concentration of 8×10^5 viable cells/ml., and confluent cell cultures were maintained in mixture '199' without serum.

THE VACCINATION TRIALS

In 1967 fifteen medical students aged 21 to 23 volunteered to participate in a comparison of inactivated and live virus B vaccines. Eight students were inoculated intranasally with live influenza B virus vaccine and seven were given inactivated vaccine subcutaneously. Nasal secretions were collected before and for 21 days after immunization. Serum was collected from each student before immunization, and at 1, 3 and 23 weeks after immunization. Stored specimens (-20°C .) were tested for H.I. and C.F. antibodies.

In 1968 a further study of similar vaccines in 39 students, who had not taken part in the previous trial, was completed by a challenge inoculation of the live vaccine virus B intranasally. There were four vaccine groups. Eleven students received intranasal live B/Eng/13/65 virus in the same dose and of the same batch as that used in 1967 and 11 received inactivated B vaccine prepared from the same strain of virus and given as a coarse intranasal spray in a 1 ml. dose. Eight students received the inactivated B vaccine subcutaneously and nine received subcutaneously an inactivated vaccine prepared from an A2 virus. The latter served as controls to the students receiving B vaccines. One month after immunization all volunteers received a challenge intranasal inoculation of 1.0 ml. of the live B virus vaccine diluted in Hanks's saline to contain $10^{6.4}$ egg infective doses (EID 50). Nasal washings were obtained before and at intervals after immunization and challenge. Sera were collected before immunization, 3 weeks after immunization, and 3 weeks after challenge. They were stored at -20°C . and tested as above.

The results of immunization

Table 1 shows that in the 1967 trial six of eight volunteers inoculated with live virus B became infected, as shown by a fourfold or greater rise in antibody. Virus was recovered from four of the six persons. Neutralizing antibody was not detected in nasal secretion before immunization in any person but in those who became infected with live virus it was detected from the 8th to the 18th day after inoculation. Five of the seven volunteers given subcutaneous vaccine developed

a fourfold or greater rise in serum H.I. antibodies yet neutralizing antibody in nasal secretions was not demonstrated during the 21 days of observation.

Table 2 shows the results obtained in 1968, which differed from those in 1967. A fourfold or greater rise in serum H.I. antibodies to influenza B occurred in seven of eight persons receiving inactivated virus B vaccine subcutaneously, in

Table 1. *Serum and nasal antibody responses in volunteers immunized with live and inactivated influenza B/Eng/13/65 virus vaccines: 1967 study*

Immunization group	No. of volunteers	Evidence of immunization		No. showing nasal antibodies when tested against 10 TCID 50	
		Virus isolation	No. showing fourfold rise in HI antibodies	Before immunization	After immunization
B/Eng/13/65. Live I.N.	8	4	6	0	6*
B/Eng/13/65. Inactivated S.C.	7	—	5	0	0

* Five of these specimens neutralized 100 TCID 50 of virus.

Table 2. *Serum and nasal antibody responses in volunteers immunized with live and inactivated influenza virus vaccines: 1968 study*

Immunization group	No. of volunteers	No. showing fourfold rise in HI antibodies	No. showing nasal antibodies before and after immunization when tested against			
			10 TCID 50		100 TCID 50	
			Before	After	Before	After
B/Eng/13/65. Live I.N.	11*	1	2	7	1	4
B/Eng/13/65. Inactivated I.N.	11	1	1	8	0	2
B/Eng/13/65. Inactivated S.C.	8	7	3	8	1	4
A2/Eng/1/61. Inactivated S.C.	9	0	2	6	0	1

* No virus was reisolated from any person.

one of each of the groups of 11 persons receiving live or inactivated influenza B virus intranasally and in none of the nine persons who received A2 vaccine subcutaneously.

Neutralizing antibodies to virus B were found in the nasal secretions of eight of the 39 volunteers in the various groups before immunization and in 29 of the volunteers on one or more occasions during the 28 days after immunization. Washings which neutralized 100 TCID 50 were found in only two persons before and in 11 after including four of 11 persons given live virus intranasally and four of eight persons receiving inactivated B vaccine subcutaneously. Eight of 11

persons receiving inactivated vaccine intranasally had nasal washings after immunization with inhibitory effects upon 10 TCID 50 and two inhibited 100 TCID 50 of virus.

Two of the nine persons immunized with A2 vaccine had nasal washings inhibitory to 10 but not to 100 TCID 50 before immunization. After immunization, which had failed to increase serum antibodies to virus B, six of the nine persons had nasal washings inhibitory to 10 TCID 50 of virus B but only a single specimen from one of these also inhibited 100 doses of virus. This unexpected result could not be readily explained unless virus B had spread from one of the volunteers given live virus intranasally. As none of these 11 persons yielded viruses by direct test of throat swabs this possibility seemed less likely than others such as a nasal non-specific inhibitor other than antibody with weak antiviral properties or a non-specific boosting of local antibody formation. Inhibition of 100 TCID 50 of virus by nasal washings was thought to be unlikely to be due to other than neutralizing antibody and such a property was generally found in consecutive nasal washings from the persons immunized with various B vaccines and not in just a single specimen as in the case of the A2 vaccinated group.

The effects of challenge of the 1968 volunteers

Table 3 shows the results of the challenge inoculation of all the volunteers with live B virus intranasally on the 28th day after immunization and their nasal antibody status at this time. The dose of virus was increased to 10^{6.4} EID 50 because

Table 3. *Immune status of immunized volunteers before and after challenge with live influenza B/Eng/13/64 virus: 1968 study*

Immunization group	No. of volunteers	No. showing neutralizing activity in nasal secretion within 16 days before challenge		No. showing evidence of infection by challenge virus by	
		Against 10 TCID 50 virus	Against 100 TCID50 virus	Virus isolation	Fourfold rise in HI antibodies
B/Eng/13/65. Live I.N.	11	5	4	1	2
B/Eng/13/65. Inactivated I.N.	11	7	2	1	2
B/Eng/13/65. Inactivated S.C.	8	8	3	1	0*
A2/Eng/1/61. Inactivated S.C.	9	6	0	0	3†

* Rise of C.F. antibodies in one person.
† Three-fold rise of H.I. antibody in one other person.

the previous dose of 10^{5.4} EID 50 given to volunteers had failed to yield virus from throat swabs. Nine of the 39 students experienced a common cold-like illness after inoculation with the larger dose of virus. Virus was reisolated from three persons, each of whom developed a fourfold or greater rise in serum antibodies by either

Table 4. Serum titres and neutralizing activity in nasal secretion of susceptible volunteers before challenge with live influenza B/Eng/13/65 virus: 1968 study

Immunization group	Total no.	Susceptible persons	Serum and nasal secretion antibody within 16 days prior to challenge			Evidence of infection with challenge virus	
			Serum antibodies (H.I.)	Neutralizing activity nasal secretions		Virus isolation	Rise in serum H.I. antibodies (4 × or more)
				10 TCID ₅₀ virus	100 TCID ₅₀ virus		
B/Eng/13/65. Live I.N.	11	J.	1/12	+	+	-	+
		R.	1/9	0	0	+	+
B/Eng/13/65. Inactivated I.N.	11	P.	<1/6	+	0	-	+
		L.	<1/6	+	0	+	+
B/Eng/13/65. Inactivated S.C.	8	M.	1/36	+	0	+	0*
A2/Eng/1/61. Inactivated S.C.	9	L.	1/9	0	0	-	+
		I.	1/9	+	0	-	+
		C.	<1/6	+	0	-	+
		E.	1/18	+	0	-	+

* Rise of C.F. antibodies × 4.

† Rise of H.I. antibodies × 3.

Table 5. *Serum titres and neutralizing activity in nasal secretions of volunteers to infection by challenge with live influenza B/Eng/13/65 virus: 1968 study*

Immunization group	Total no. of volunteers	No. resistant to infection	Serum antibodies (H.I.) within 16 days prior to challenge					Neutralizing activity nasal secretions				
			within 16 days prior to challenge					Within 16 days prior to challenge				
			< 1/6 1/6-1/18 > 1/24					10 50 100 TCID 50 virus				
								10 50 100 TCID 50 virus				
B/Eng/13/65. Live I.N.	11	9	2	1	6			4				4
B/Eng/13/65. Inactivated I.N.	11	9	2	5	2			5				3
B/Eng/13/65. Inactivated S.C.	8	7	0	1	6			7				5
A2/Eng/1/61. Inactivated S.C.	9	5	1	2	2			3				2

H. I. or C.F. test. Five more developed a fourfold increase in H.I. antibodies, and one showed a threefold antibody rise. Four volunteers with serological evidence of infection belonged to the control group originally given A2 vaccine. Altogether nine infections were judged to have occurred among the 39 immunized persons (Table 4).

Fig. 1. Diagrammatic representation of serum and nasal antibodies in immunized volunteers before attempted infection with intranasal live B/Eng/65 virus.

Table 4 shows the H.I. serum titres just before challenge and also the presence or absence of neutralizing antibody in nasal secretions collected during the previous 16 days before challenge for each of the nine volunteers. Three persons had no detectable serum antibodies at a 1/6 dilution and only one had a serum titre in excess of 1/24. Neutralizing antibody was not detected in nasal secretions from two of the volunteers but was detected in seven against 10 TCID 50 of virus and in one against 100 TCID 50 as well.

The results in those who resisted challenge by neither yielding virus from throat swabs nor developing a fourfold or greater increase in serum antibodies are shown in Table 5 grouped together by method of immunization. Out of 30 resistant volunteers, 16 (53%) possessed serum H.I. antibody titres of or in excess of 1/24 and only five failed to inhibit haemagglutination at 1/6 dilution of serum. Inhibitory activity was present against 10 TCID 50 of virus in the nasal secretion before challenge in 19 persons and was active against 100 TCID 50 of virus in eight instances. Serum H.I. antibody was present in a titre of or in excess of 1/24 in 12 persons in whom antibody was also present in nasal secretion. Of these 12 persons,

the nasal washings from six inhibited 100 TCID₅₀ of virus, and from six more 10 TCID₅₀. Among the 14 whose serum titre was less than 1/24, neutralizing activity was present in the nasal secretion in seven but was only active against 100 TCID₅₀ in two persons.

These results are presented diagrammatically in Fig. 1, which compares serum antibodies and nasal washings inhibitory to virus at the time of the challenge infection in both resistant and susceptible volunteers. Resistance to infection was correlated to some extent with the titre of serum H.I. antibodies in that those with titres of 1/24 or more escaped infection except in one instance. Resistance was exhibited by 11 volunteers in whom nasal antibodies were not demonstrated. Four had high serum titres ($> 1/24$) but seven did not and in these some other factor may have been responsible for the resistance. Including those with nasal inhibition of only 10 TCID₅₀ and those inhibiting 100 TCID₅₀ of virus, there were seven of 26 persons with nasal inhibition who contracted infection but all but one of these had low serum antibody levels less than 1/24. Most of the 19 other persons, who resisted infection, had demonstrable serum antibodies as well as nasal inhibitory substances but two had none.

DISCUSSION

It has been known for 25 years that inactivated influenza virus vaccines will confer a significant degree of immunity against influenza in the field (Report of the Medical Research Council Committee, 1958; Davenport, 1961). This immunity has traditionally been believed to be mediated by antibody formation and the recent experimental work on small numbers of persons confirms that these antibodies appear after subcutaneous inoculation in both serum and nasal secretions (Mann *et al.* 1968; Kasel *et al.* 1969).

Waldman *et al.* (1968), using an inactivated A2 virus, and Kasel *et al.* (1969), using a polyvalent vaccine, found that the intranasal route was better for inducing neutralizing antibody in nasal secretions than was the subcutaneous route. However, in 1968 we found that inactivated influenza B virus gave a better neutralizing antibody response in nasal secretions after subcutaneous inoculation than after intranasal immunization. The poor response in nasal secretions after intranasal vaccine may have been due to the low dose employed, since the volunteers were not given a booster inoculation.

The present study has emphasized the great difficulty in obtaining consistent results in volunteers perhaps because of changes in the immune status of the population resulting from natural exposure to infection. Thus the ready demonstration of nasal antibodies before immunization in the volunteers in 1968 compared with its absence from others in 1967 is strongly suggestive of recent exposure to influenza B virus. Moreover, the results of attempted infection with attenuated virus are also consistent with exposure to influenza B virus in the preceding months of 1968, and indeed there was a ten-fold increase in reported illnesses due to influenza B virus during the period from mid-March to mid-April 1968.

The evidence collected by workers with viruses such as parainfluenza Type I (Smith, Purcell, Bellanti & Chanock, 1966) and rhinoviruses (Cate *et al.* 1966;

Fleet, Couch, Cate & Knight, 1965) have shown that both serum and nasal antibodies are required to confer resistance to direct challenge by intranasal virus. The experiments made in Chicago with parainfluenza Type 2 virus by Tremonti, Lin & Jackson (1968) also suggest a synergistic effect of nasal and serum antibody. Our own work with influenza B supports these findings in that resistance to infection sometimes appeared to depend upon serum antibodies only but more often upon a combination of serum and nasal antibodies. Persons susceptible to infection mostly possessed weak or no inhibitory activity in nasal washings.

Infection might also have been resisted on some basis other than neutralizing antibody since three of the volunteers who were not susceptible to the challenge dose of virus lacked neutralizing antibody in both nasal secretions and serum. Gresser & Dull in 1964 described an interferon-like inhibitor in pharyngeal washings from patients with clinical influenza. In 1965 Jao, Wheelock & Jackson detected an interferon in nasal washings and serum of volunteers challenged with a live influenza A2 virus. It has also been observed that after infection with common cold-like viruses there is an increased resistance to infection by other viruses (Lidwell & Williams, 1961; Fleet *et al.* 1965). Finally, antibody to influenzal neuraminidase, although not preventing the initiation of infection, may be involved in limiting the extent of infection in the host. Schulman, Khakpour & Kilbourne (1968) have found that neuraminidase-inhibiting antibody increased the survival of mice when challenged with influenza virus.

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