

Serological studies with purified neuraminidase antigens of influenza B viruses

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(Received 2 July 1973)

SUMMARY

Neuraminidase (N) can be extracted from virus particles of influenza B strains by treatment with trypsin, in a form which is free from the viral HA and has specific immunological activity. The N antigen of B/LEE/40 behaves differently from that of 1965-6 strains in gel diffusion and enzyme inhibition tests with animal antisera raised by infection or by artificial immunization with the homologous or heterologous strains. The frequency and titres of NI antibody detected in human sera by B/LEE antigen are different from those found with antigen from B/Eng/13/65. The latter antibody appears to contribute to the effect of serum HI antibody in protecting volunteers exposed to a deliberate intranasal challenge infection of the B/Eng/13/65 strain.

INTRODUCTION

In the thirty-three years since the first isolation of influenza type B viruses (Francis, 1940) their world-wide distribution has been repeatedly observed, but they have never given rise to pandemics like those seen recurrently with influenza A viruses. The outbreaks tend to be localized and to affect children more commonly than adults in whom individual illnesses may be so mild as to pass unrecognized as anything more than a 'cold' unless serological investigations are performed. This may mean that type B viruses inherently lack virulence or epidemicity, but it is possible that exposure to any one strain may confer broad protection against subsequent strains or reduce the clinical effects of re-infection, as occurs with the parainfluenza and respiratory syncytial myxoviruses. The infectivity of type B virus is significantly reduced in volunteers with high pre-challenge titres of serum HI antibody against the homologous virus haemagglutinin (HA) (Hobson, Curry, Beare & Ward-Gardner, 1972). However, there has been considerable antigenic drift in the HA of strains isolated between 1940 and 1972 (Chakraverty, 1972*a*), and it seems unlikely that HI antibody against the prototype B/LEE strain of 1940 would have any protective value against present-day strains. It thus seems pertinent to investigate whether any one influenza B infection might induce widely reactive antibody against a type-common neuraminidase (N), which could

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contribute to the short-term effect of relatively strain-specific HI antibody and might play a major role in long-term immunity to type B influenza.

The role of N antigens has been less thoroughly investigated for type B than for type A viruses, largely because of technical problems. Segregation of HA and N antigens by genetic recombination is relatively easy for type A, but has not been achieved with type B. The use of suspensions of whole virions, containing both HA and N antigens, to measure NI antibody in postinfective serum tends to show false enzyme inhibition because of steric hindrance by homologous HI antibody also present in the serum. Recently the treatment of various type B strains with trypsin by a modification of the method of Noll, Aoyagi & Orlando (1962) has allowed both HA and N antigens to be separated in high yield (Brown, Hobson & Curry, 1973) and the present paper reports studies with partially purified N to investigate the NI responses of experimental animals and man after infection with various type B viruses and the possible role of NI antibody in conferring resistance to influenza B.

MATERIALS AND METHODS

The derivation and cultivation of all the virus strains used have already been described (Brown *et al.* 1973).

For the preparation of separate HA and N antigens, virus was recovered from infected allantoic fluids by centrifugation, washed in phosphate buffered saline (PBS A) and concentrated to 6000 HA units per ml. Trypsin (Difco 1/250) was added to a final concentration of 10 mg./ml. and after incubation with constant mixing at 37° C. for 45 min. the reaction was stopped by the addition of soybean trypsin inhibitor (BDH) to a final concentration of 10 mg./ml. Centrifugation of the mixture at 40,000 *g* for 1 hr. yielded a deposit containing virus particles with HA activity only, whereas the supernatant contained all the enzyme activity of the original virus suspension but was devoid of HA. Before use in the tests described below, N was separated from other viral material, trypsin and soybean extract by passage through a 1.5 × 20 cm. column of Sephadex G75 (Pharmacia). Enzyme activity was recovered quantitatively in the first of four protein peaks, which was shown by polyacrylamide gel electrophoresis to contain a single protein.

The enzyme was assayed by the Warren method with fetuin substrate, and NI titrations of sera were made with freshly prepared batches of separated enzyme; details of the methods are as described by Hobson *et al.* (1972).

Rabbit antisera were obtained by intramuscular inoculation at multiple sites with column-purified N (the amount obtained from 3000 HA units of virus) emulsified in an equal volume of complete Freund adjuvant (Calbiochem); the animals were bled 4 weeks later. Guinea-pig postinfective sera were obtained by cardiac puncture of 8-week-old animals 14 days after the second of two monthly intranasal instillations of 10⁴ EID₅₀ (egg infective doses) of the appropriate virus strain in 0.05 ml. of PBS A; animals were inoculated under light ether anaesthesia. All procedures and housing were in separately ventilated quarantine rooms to avoid cross-infection. No clinical illness was noticed in any animal. No HI or NI activity was found in preinfective sera from these guinea-pigs.

Table 1. *The specific activity of B/LEE virus fractions obtained by treatment with trypsin*

	HA titre per ml.	N* titre/ml.	Serum titres of rabbits twice inoculated i.v. with 1 ml. of the fraction in soluble form	
			HI	NI†
Whole virus	5120	100	3072	500
Trypsinized virus	{ Deposit‡	2560	1536	15
	{ Supernatant‡	< 2	12	300

* Dilution of fraction which gives an optical density of 0.5 in the Warren test after release of NANA from 4.8 mg. fetuin substrate.

† Dilution of serum which gives 50% inhibition of this standard dose of N.

‡ Fractions were reconstituted to the original volume of whole virus suspension.

The volunteer experiments from which human sera were derived, virus challenge procedures and scoring of infection rates were as detailed by Beare, Hobson, Reed & Tyrrell (1969) and Hobson *et al.* (1972).

RESULTS

Separation of HA and N antigens

B/LEE virus was treated by the procedure described above. As shown in Table 1, viral HA apparently was recovered quantitatively with the virus particles after high-speed centrifugation; this deposit fraction had no detectable N enzymic activity. Inoculation into rabbits gave high HI titres but very little NI antibody. Conversely, the supernatant fraction had no detectable HA, but contained all the enzymic activity of the original virus suspension: rabbit antisera showed high NI titres but only low titres of HI antibody. It is probable that the small cross-activity of each fraction represents residual contamination which might be removable by more extensive purification procedures.

Serological responses of infected guinea-pigs

Pairs of animals were infected with B/LEE, B/Tai/4/62 or B/Ann Arbor/1/66 which are known to show large differences in their HA antigens. Convalescent sera were titrated with the separated HA and N antigens of each virus (Table 2). The HA antigens detected an entirely monospecific homologous HI response. B/LEE N demonstrated NI antibody only in homologous serum. The N antigens of B/Tai and B/Ann Arbor were inhibited to some degree by all the sera, but those from the homologously infected animals gave the highest titre; both of these antigens were considerably less effective in detecting NI antibody in B/LEE-infected guinea-pigs than was the homologous B/LEE N.

The cross-reactivity of rabbit antisera

Even after a more artificial and intensive immunization procedure than was used for the guinea-pigs, the N fractions of the three viruses again showed antigenic

Table 2. *The serological response of guinea-pigs after intranasal infection with 10⁴ EID₅₀ of influenza B viruses*

Test	Source of antigen	Serum antibody titres in guinea-pigs infected with		
		LEE	TAI	AA
HI	B/LEE/40	384	< 6	< 6
	B/TAI/4/62	< 6	192	< 6
	B/Ann Arbor/1/66	< 6	< 6	1536
NI*	B/LEE/40	640	< 10	< 10
	B/TAI/4/62	160	750	400
	B/Ann Arbor/1/66	25	600	640

* Titre = the dilution of serum which gives 50% inhibition of that dose of N which produces an optical density of 0.5 at 549 nm. (NANA release) from 4.8 mg. fetuin in the Warren test with normal rabbit serum.

Table 3. *The reaction of purified N from three type B virus strains with the corresponding immune rabbit antisera*

Source of N	NI titre* of antiserum raised against		
	LEE	TAI	13/65
B/LEE/40	500	300	20
B/TAI/4/62	120	2000	180
B/Eng/13/65	120	1000	400

* The measurement of titres is as described in Table 2.

heterogeneity in the NI test with rabbit antisera (Table 3) and in gel diffusion tests with the various antigens (Plate 1) B/LEE N appeared distinct from the N of the two more recent viruses.

The distribution of NI antibody in normal human sera

Sera from 92 unvaccinated adults with no recent history of influenza were titrated with the separated HA and N of B/Eng/13/65 virus, which is closely related antigenically to the B/Ann Arbor strain. NI antibody was detected in 87 (94%) sera (Fig. 1), but HI antibody was present in only 55 (60%). There was little correlation between the two titres, except that in general those with high HI titres also showed high NI titres. However, in those with no HI antibody, NI titres were found over a range of 1/50–1/500.

Sera from 80 unvaccinated normal adults were tested at a single dilution of 1/100 for their enzyme-inhibitory activity against comparable standardized doses of N fractions of B/LEE and B/Eng/13/65. Most sera (Fig. 2) showed NI activity against both antigens, but there was no apparent correlation between the two; the results suggest that each antigen might be measuring a separate and distinct NI antibody.

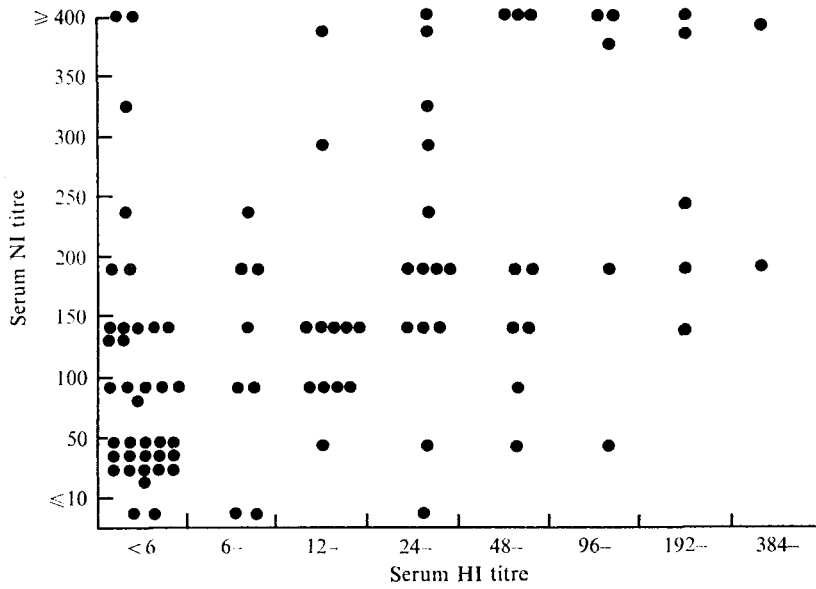


Fig. 1. The distribution of HI and NI serum antibody titres in 92 unvaccinated adults.

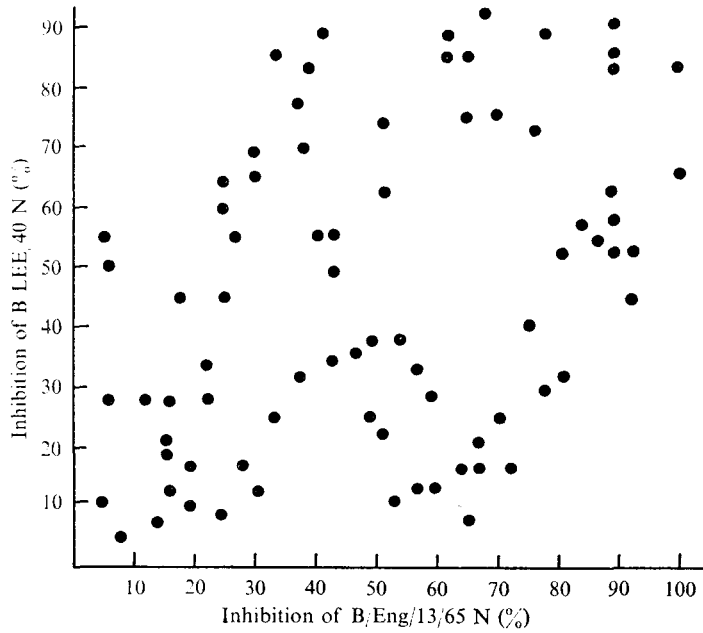


Fig. 2. The serum NI activity of 80 normal adults measured with the N antigen of two different influenza type B viruses. Each serum was used at a single dilution of 1/100 in the NI test (see text).

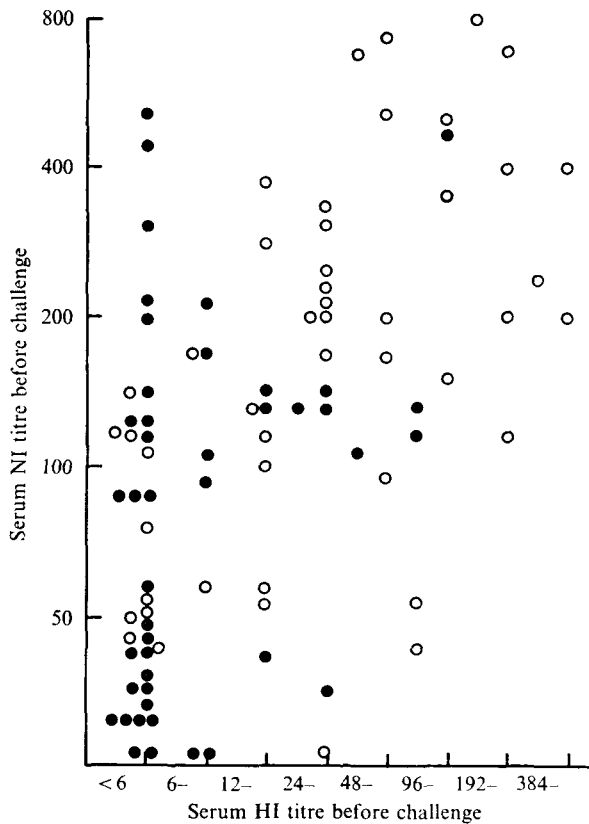


Fig. 3. The relation of serum HI titre and serum NI titre to protection against subsequent challenge infection of 91 volunteers with the homologous B/Eng/13/65 virus. Result of challenge: ● = infected; ○ = not infected.

Table 4. *The effect of pre-existing HI and NI serum antibodies on infection in 91 volunteers challenged with the homologous influenza B virus*

	Pre-challenge immune status of volunteer			
	High HI High NI	High HI Low NI	Low HI High NI	Low HI Low NI
Total no.	15	6	22	47
No. infected	1	2	10	31
Infection rate %	7	33	43	66

High HI \geq 48. High NI \geq 150.

The relation of NI antibody to protection against influenza

Ninety-one randomly chosen volunteers were given a challenge intranasal spray of B/Eng/13/65 virus immediately after a sample of serum had been obtained for HI and NI titrations against the homologous virus. The distribution of proved infections (Fig. 3) was predominantly in those with low pre-existing serum HI titres, but it also seemed that those with low titres of NI antibody were more susceptible to influenza than those with titres of 1/150 or greater. However, further

analysis of these results (Table 4) suggest that those with high titres of NI antibody alone were less well protected than those with high HI titres alone, whilst those with high titres of both antibodies showed the lowest infection rate after challenge.

DISCUSSION

The influenza B viruses isolated from 1940 onwards do not fall into distinct subtypes of different temporal incidence or in different species of host animal, as do the influenza A strains. Although their HA antigens show considerable diversity, this is generally regarded as antigenic drift within a single type rather than antigenic shift between subtypes, as seen in type A (Pereira, 1969; Chakraverty, 1972*a*). Thus, until recently it was believed that the viral neuraminidase would be likely to have a single type-common antigenic constitution; however, Chakraverty (1972*b*) found that animal antisera raised against a variety of type B strains may show widely differing NI titres according to the virus strain employed in the test. Our present findings provide further evidence of antigenic heterogeneity of type B_N; in particular the prototype B/LEE/40 differs sharply from more recently isolated strains in the reactivity of enzyme inhibitory antibody it can induce or detect.

In earlier attempts, such as our own (Hobson *et al.* 1972), to define the role of NI antibody in protection against type B influenza, measurement of preinfective titres with strains other than the homologous challenge virus could lead to unduly pessimistic conclusions. We had previously shown that NI titres against B/LEE did not bear any significant relation to immunity in adult volunteers given a deliberate challenge infection with B/Eng/13/65 virus, whereas it is clear from the present study that pre-challenge NI titres measured by B/Eng/13/65 are of some predictive value in relation to the result of subsequent exposure to the homologous strain, as was suggested for influenza A strains by Slepshkin *et al.* (1971).

Since the N antigens of type B are apparently not homogeneous, it seems unlikely that NI antibody against one strain would confer long lasting protection against the epidemic spread of strains emerging decades later. However in the short term, NI antibody against current strains related to B/Eng/13/65 may add to the protective effect of homologous serum HI antibody in volunteers where both are present, and may be important in protecting those without HI antibody. The greater frequency of NI than HI antibodies in the present panel of volunteers might merely reflect the greater sensitivity of the NI titration, but it is possible that N antigen has changed less frequently than HA over the last 4–8 years; repeated exposure during this time may thus recall or maintain effective NI antibody levels more commonly than HI antibody, and thus offset to some extent the effect of antigenic drift.

This study was carried out with the support of a research grant from the Medical Research Council. It is a pleasure to express our indebtedness for this generous assistance.

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EXPLANATION OF PLATE

Plate 1. The reactivity of rabbit antiserum, raised by the isolated N antigen of B/Eng/13/65 virus, in gel diffusion against isolated N antigens of four type B strains. The four virus N antigens were obtained from: B/LEE/40 (LEE); B/Taiwan/4/62 (TAI); B/Ann Arbor/1/66 (A.A.); B/Eng/13/65 (13/65).

