

## **Quantitation and analysis of the specificity of post-immunization antibodies to influenza B viruses using single radial haemolysis**

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### SUMMARY

The single radial haemolysis (SRH) technique detected anti-B/HK/8/73 HA antibody rises in 59–85% of paired sera from persons immunized with different influenza vaccines. In contrast, analysis of the same sera by the haemagglutination inhibition (HI) test indicated significant antibody rises in only 27–54% of paired sera. High levels of antibody were detected to influenza B/HK/8/73 and B/Singapore/222/79 viruses in post-immunization sera analysed by SRH, whereas the HI test indicated comparatively low geometric mean antibody titres. Most adults responded to immunization with influenza B virus by producing cross-reactive (CR) antibody which reacted with different influenza B viruses including the early isolate B/Lee/40.

### INTRODUCTION

Studies on the epidemiology of influenza B virus have been hampered by the low sensitivity of the standard haemagglutination inhibition (HI) test (La Montagne, 1980; Monto & Maassab, 1981). Moreover, HI antibody titres to influenza B viruses have been shown to be an unreliable index of immunity (Wright, Bryant & Karzon, 1980). Chakraverty (1980) has described the use of the single radial haemolysis (SRH) test for sero-epidemiological studies with influenza B viruses isolated in 1973 and 1975, and the technique appeared to be more sensitive than the HI test. We describe here the application of a modified SRH technique (Oxford *et al.* 1981) to quantitate and to analyse the specificity of the anti-haemagglutinin (HA) antibody response following vaccination of adults with influenza B viruses including B/Hong Kong/8/73 and the recent antigenic variant B/Singapore/222/79. The SRH test is shown to be significantly more sensitive than the HI test for the detection of both post-vaccination levels of anti-HA antibody and seroconversions to influenza B viruses.

## MATERIALS AND METHODS

*Viruses*

Influenza A and B viruses were grown in embryonated hen's eggs by standard procedures. Concentrated and purified virus preparations were prepared as described previously (Skehel & Schild, 1971).

*Antisera*

Hyperimmune sera were prepared by immunizing sheep or rabbits with influenza B virus HA enzymically released from purified virus using bromelain enzyme (Brand & Skehel, 1972).

Animals were injected intramuscularly with approximately 50  $\mu\text{g}$  of virus HA in 1.0 ml PBS together with an equal volume of Freund's complete adjuvant. Serum samples were analysed by immunodouble diffusion (Schild, Oxford & Virelizier, 1976) after 2 weeks and, if necessary, the animals were given a booster dose of HA and bled approximately a week later. Post-infection ferret sera were prepared using conventional techniques (Stuart-Harris & Schild, 1976).

*Inactivated vaccines and immunization schedules*

Students (University of Nottingham) were immunized intramuscularly (i.m.) with 0.5 ml of different trivalent whole virus or subunit vaccines containing A/England/321/77 (H3N2), A/USSR/92/77 (H1N1) and B/HK/8/73 viruses. The HA concentration ( $\mu\text{g}$ ) of the B/HK/8/73 component is detailed in Table 1. Sera were taken 14 days post-immunization. In a further trial, adults were immunized i.m. with a trivalent Tween-ether split vaccine (Merieux) containing B/Singapore/222/79, A/Brazil/11/78 (H1N1) and A/Bangkok/1/79 (H3N2) viruses. Sera were taken 23 days post-immunization.

*Single radial haemolysis (SRH) test and virus adsorption experiments*

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). Purified virus was added (10  $\mu\text{g}$  virus protein per ml of 10% erythrocyte suspension) and within 10 min at 4 °C a visible haemagglutination commonly occurred and a half volume of the freshly diluted  $\text{CrCl}_3$  solution was then added. The modification of the SRH test (Schild, Pereira & Chakraverty, 1975) to include chromium chloride has been shown by electron microscopy to result in a more permanent and a higher rate of attachment of virions to red blood cells (D. Hockley, personal communication). The virus-RBC mixture was allowed to stand at 4 °C for 5 min with occasional mixing. The cell suspension was sedimented by gentle centrifuging (1000 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) and once in phosphate-buffered saline (PBS) pH 7.2 containing 0.2% BSA. For influenza B viruses a limited chequerboard titration was performed, and the

relative proportions of virus-sensitized red blood cells to guinea-pig complement varied by up to twofold to obtain optimum haemolysis zones. Generally, immunoplates contained 0.3 ml of virus-sensitized red blood cells (10% v/v) and fresh guinea-pig complement (0.15 ml) in agarose gel (2.5 ml) and were prepared as described previously (Schild, Pereira & Chakraverty, 1975; Oxford *et al.* 1981). The SRH plates or virus-sensitized red blood cells could be stored at 4 °C for several weeks before use.

Sera were heated at 56 °C for 30 min to destroy complement, and 10  $\mu$ l volumes added to the wells of the SRH plates. The plates were incubated in a humid atmosphere at 37 °C and the zones of haemolysis developing after 4 h and 18 h were measured using a calibrating viewer (Transdyne General Corporation). An increase in SRH zone area of 50% was considered to indicate a significant rise in antibody titre as described previously for influenza A viruses (Oxford *et al.* 1979).

In certain experiments to establish whether post-vaccination sera contained CR or SS antibodies to the different influenza B viruses, undiluted sera (20  $\mu$ l) were adsorbed with approximately 6  $\mu$ l of concentrated virus (10 mg/ml protein of B/Lee/40, B/HK/8/73 or B/Singapore/222/79 viruses) for 30 min at room temperature before addition to the wells of the SRH plates containing the different viruses (Oxford *et al.* 1979). Preliminary experiments established that the quantity of influenza B virus used for adsorption in these experiments was in excess of that required to adsorb the SS or CR antibody. CR and SS are operational terms and are used as defined previously (Schild *et al.*, 1977; Oxford *et al.*, 1979).

#### *Haemagglutination inhibition (HI) test*

Sera were treated with receptor-destroying enzyme (RDE, Phillips Duphar) by overnight incubation at 37 °C to remove non-specific inhibitors. The sera were heated at 56 °C for 30 min to destroy the RDE and titrated in twofold dilution steps versus 8 HA units of influenza B viruses by the conventional HI test (Stuart-Harris & Schild, 1976). A fourfold or greater rise in antibody titre was considered to indicate a significant rise.

## RESULTS

The anti-HA antibody responses estimated by HI or SRH tests and following immunization of young adults with different whole influenza virus or subunit vaccines containing B/HK/73 virus are shown in Table 1. A relatively poor serological response was detected using the HI test, since a fourfold rise in antibody titre was detected in a relatively low proportion of vaccinees (between 27 and 54%). In contrast, analysis of the sera by SRH demonstrated that between 59 and 85% of persons produced a significant antibody response following immunization. Moreover, the quantitative response, measured by zone areas of haemolysis (Plate 1) was satisfactory and comparable (range from 5.4 to 12.8 mm<sup>2</sup> SRH zone area) to the homologous SRH response following vaccination with influenza A viruses (data not presented).

Table 1. Comparison of HI and SRH techniques for the quantitation of post-immunization antibody in adults to the homologous virus in recipients of B/HK/73 and B/Singapore/222/79 vaccines

Vaccine	HA ( $\mu$ g) per dose (0.5 ml)	No. of vaccinees	Percentage with significant* antibody rises		HI (GMT)		SRH (mean area in mm <sup>2</sup> )	
			HI	SRH	pre	post	pre	post
B/HK/8/73								
Trivalent, whole virus	35.7	26	54	85	< 10	35.0	< 0.3	12.8
Trivalent, whole virus	9.4	22	32	59	< 10	24.7	< 0.3	7.0
Trivalent, HA, NA, subunit	20.2	26	27	62	< 10	15.6	< 0.3	5.4
Trivalent, HA, NA, subunit with aluminium hydroxide adjuvant	ND	26	46	65	< 10	20.6	< 0.3	5.4
B/Singapore/222/79								
Tween-ether split	11.5	23	35	68†	< 10	27.9	< 0.5	11.8

\* Significant rise is fourfold increase in HI titre or 50% increase in SRH zone area.

† Nineteen sera analysed only.

Pre and post: pre- and post-immunization.

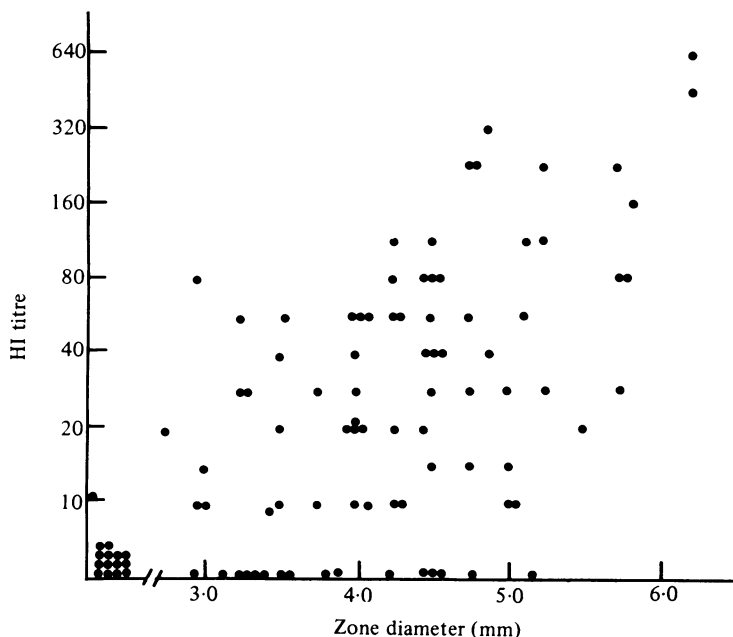


Fig. 1. Correlation between HI titres and SRH zone diameter in post-immunization sera from adults. Anti-HA antibodies in sera from adults immunized with different B/HK/8/73 virus-containing vaccines were analysed by HI or SRH techniques as detailed in the text.

Fig. 1 shows the relatively poor correlation between individual HI titres for 99 of the post-immunization sera and the corresponding SRH zone diameter. A proportion of the sera (15%) gave SRH zone diameters between 3.0 and 5.2 mm<sup>2</sup> but had no detectable level of HI antibodies. Fourteen sera had no antibody detectable by either of the techniques. The correlation coefficient calculated for positive values shown in Fig. 2 was 0.3, which was statistically significant but low value. This indicates an underlying but weak relationship between SRH zone diameters and HI titres for the influenza B viruses tested.

More recently a new antigenic variant of influenza B virus, B/Singapore/222/79, has been isolated and the current influenza vaccines contain this virus (Weekly Epidemiological Record, 1980). It was of interest, therefore, to analyse the antibody response to vaccine containing B/Singapore/222/79 virus in comparative tests using HI and SRH techniques. As noted above with B/HK/8/73 virus, the SRH test detected relatively higher levels of post-immunization antibody and also detected a higher proportion of sero-conversions than was detected by the HI test (Table 1). Thus 68% of volunteers responded to immunization and produced a significant antibody rise when the sera were tested by the SRH method, compared to 35% responding by the HI test. Similarly the geometric mean HI titre following immunization with B/Singapore/222/79 virus was low (27.9) compared to a GMT of 191.7 which was obtained with the A/Brazil/11/78 (H1N1) virus component of the same vaccine. The mean zone area for the post-immunization sera estimated

by SRH following immunization with B/Singapore/222/79 virus was 11.8 mm<sup>2</sup>, and thus compared satisfactorily to a mean zone area of 6.1 mm<sup>2</sup> following immunization with the A/Brazil/11/78 (H1N1) virus component in the same vaccine.

*Analysis of post-immunization sera for SS and CR antibodies to the HA of influenza B viruses*

The SRH test has been used previously to analyse the specificity of the antibody response to vaccines containing influenza A viruses (Schild *et al.* 1977; Oxford *et al.* 1981). Most adults respond to immunization by producing cross-reactive (CR) antibodies which react with the HA of all viruses of a single antigenic subtype.

Comparative analysis of a proportion of the sera in the present study indicated that most adults (89%) responded to immunization with B/HK/8/73 virus by producing CR antibody which reacted with both B/Lee/40 and the homologous virus. SS antibody was also detected in a relatively high proportion (67%) of adult sera (Table 2).

#### DISCUSSION

In the present paper we have demonstrated the greater sensitivity of the SRH technique compared to the HI method in the detection of post-immunization antibody to influenza B viruses, including the most recent antigenic variant B/Singapore/222/79 virus. The SRH test detected more post-immunization rises in antibody, compared to the HI test, in persons immunized with whole virus vaccines, subunit vaccines or subunit vaccines adsorbed onto an aluminium hydroxide carrier. It is most likely that the greater sensitivity of the SRH test described here results from the increased reproducibility of the test allowing the reliable detection of small rises in antibody (50% increase in zone area). In contrast, the much greater experimental variability of the HI test means that only fourfold or greater rises in antibody titre can be accepted as significant. Recently, a modification of the HI test for influenza B viruses using ether-treated antigens has been described which is more sensitive than the conventional HI test (Monto & Maassab, 1981) for detecting post-infection rises in antibody. The comparative sensitivity of the latter test and the SRH technique is not known at present, nor is the relationship between antibody titres detected using these tests and immunity to infection with influenza B viruses. However, with both influenza A and B viruses we consider that the SRH test is more accurate and reproducible than the HI test for the detection of anti-HA antibody (Schild, Pereira & Chakraverty, 1975; Schild *et al.* 1977). In addition the SRH plates may be stored for several weeks before use and the sera do not have to be diluted or treated to remove non-specific inhibitors.

Previous studies have established antigenic relationships between the HA of influenza B viruses isolated between 1940 and 1973 using HI (Chakraverty 1971, 1980; Luzyanina *et al.* 1979) or immunodouble-diffusion tests (Schild *et al.* 1973). Only a relatively few strains of influenza B virus have been analysed in the present study, but the viruses include the most recent antigenic variant of epidemiological

Table 2. Specificity of anti-influenza B HA antibodies in pre-and post-immunization sera

Adults	No. of sera with following antibody									
	SS <sub>Lee</sub>			SS <sub>HK</sub>			CR			
	No. of sera tested	Pre	Post	Percentage rises	Pre	Post	Percentage rises	Pre	Post	Percentage rises
	18	0	1	6	4	14	67	5	18	89

Sera were adsorbed with excess purified B viruses and tested on the relevant SRH immunoplates as described in Materials and Methods. SS<sub>Lee</sub> = strain-specific antibody to HA of B/Lee/40-virus; SS<sub>HK</sub> = strain-specific antibody to HA of B/HK/8/73 virus; CR = cross-reactive antibody to HA of both B/Lee/40 and B/HK/8/73 viruses. Thus sera with CR antibody produced haemolysis in both immunoplates containing B/Lee/40 virus and B/HK/8/73 virus. In addition, CR antibody could be removed by adsorption with either virus. Sera which, after adsorption with B/HK/8/73 virus, CR antibody could be removed by immunoplate possessed SS<sub>Lee</sub>/40 antibody. Similarly, sera which reacted only on the B/HK/8/73 immunoplates possessed SS<sub>HK</sub> antibody and this could be removed by adsorption only with B/HK/8/73 virus.

importance, B/Singapore/222/79. The HA of the influenza B viruses tested appear to form a single antigenic subtype with respect to the HA antigen (unpublished data).

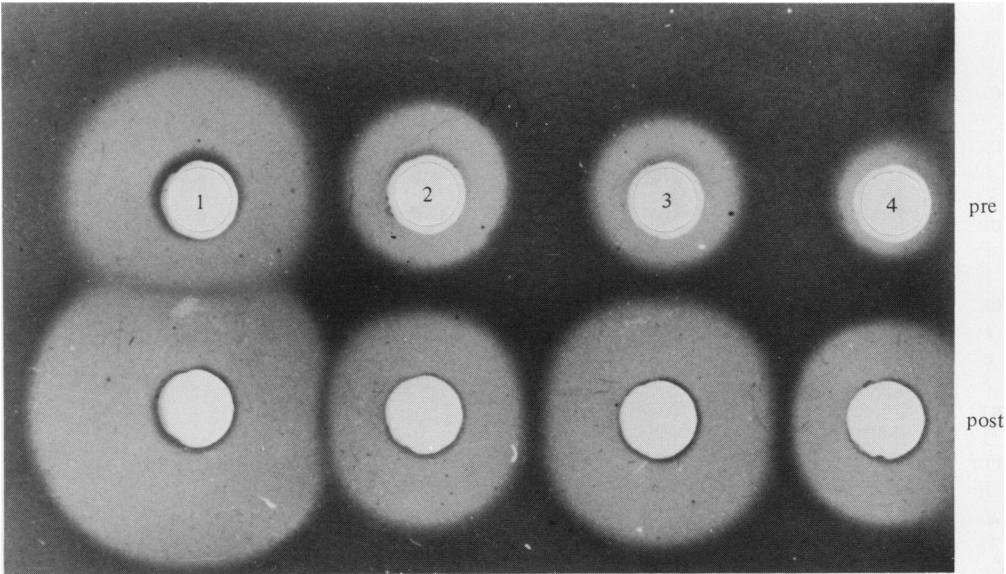
Preliminary analysis of the specificity of SRH antibody induced following immunization with influenza B viruses has demonstrated a response analogous to that observed following immunization with influenza A viruses (Schild *et al.*, 1977; Oxford *et al.* 1981; Couch *et al.* 1979). The early reported phenomenon of 'original antigenic sin' for influenza A viruses (Francis, 1953; Davenport, Hennessy & Francis, 1953; Fazekas de St Groth & Webster, 1966) may also explain, therefore, this antibody response to influenza B viruses. Adults responded to immunization by producing CR antibody which reacted with a wide range of viruses in the subtype. Presumably the relatively low proportion of adults in the present study producing SS antibody to the earliest isolated virus B/Lee/40 reflects the age range of the volunteers. Most of the sera were from persons born after 1960, when influenza B viruses which differed significantly in their antigenic composition from influenza B/Lee/40 virus were causing influenza in the community (Chakraverty, 1980). Further adsorption studies with a range of such earlier viruses would be required to analyse completely the specificity of the antibody response particularly to the SS antigenic determinant following immunization with influenza B virus.

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## EXPLANATION OF PLATE 1

Zones of haemolysis induced by pre- and post-immunization sera in SRH immunoplate containing influenza B/HK/8/73 virus. B/HK/8/73 virus-sensitized red blood cells were incorporated into agarose containing guinea-pig complement as described in Materials and Methods. Wells are numbered 1-4, and sera in the top row are pre-immunization samples and sera in the bottom row post-immunization samples.