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Interpretation of responses and protective levels of antibody against attenuated influenza A viruses using single radial haemolysis

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SUMMARY

Antibody determinations against H3N2 and H1N1 type A influenza viruses were carried out on paired sera obtained from volunteers taking part in influenza virus vaccine studies, using both the haemagglutination-inhibition (HI) and single radial haemolysis (SRH) test. Good correlation between the HI and SRH test was found for both H3N2 and H1N1 antibody and the zone area increases corresponding to significant SRH antibody rises determined for both virus strains. In both H3N2 and H1N1 vaccine studies, intranasal infection of the volunteers with live attenuated viruses was involved and by the measurement of HI and SRH antibodies prior to and following infection, levels of antibody equating with protection against the infecting viruses could be estimated. For the HI test the antibody titres associated with 50% protection were 42 for H1N1, and 44 for H3N2 viruses; for the SRH test, 50% protection was associated with zone areas of 20·0–25·0 mm^2 for both H1N1 and H3N2 viruses.

INTRODUCTION

The single radial haemolysis (SRH) test (Russell, McCahon and Beare, 1975; Schild, Pereira & Chakraverty, 1975) is now widely used for the determination of antibody to influenza A and B viruses in both vaccine studies and following natural infections (Vaananen *et al.*, 1976; Farrohi *et al.* 1977; Delem & Jovanovic, 1978; Cretescu *et al.* 1979; Chakraverty, 1980; Grilli & Davies, 1981; Oxford, Yetts & Schild, 1982; Yamagishi *et al.* 1982; Goodeve, Jennings and Potter, 1983). It is a test both simple and reproducible, rapid to perform, and more sensitive than the conventional haemagglutination-inhibition (HI) test (Russel, McCahon & Beare, 1975; Oxford *et al.* 1982; Goodeve, Jennings & Potter, 1983; Jennings *et al.* 1984). Antibody measured by SRH is expressed in terms of zone areas of haemolysis (Russell *et al.* 1975), and in recent years the zone area increase corresponding to significant rises in antibody titre in serum pairs have been defined for both influenza A (Oxford *et al.* 1982) and influenza B viruses (Goodeve *et al.* 1983).

In the present study, we confirm that a difference of 50% in zone area between two sera tested on the same immunoplate represents a significant difference in titre, for both H1N1 and H3N2 influenza virus antibodies, and also establish another parameter with respect to the SRH to test, namely the SRH antibody levels that

equate with 50 % protection against infection of man with H1N1 or H3N2 influenza virus strains.

This parameter previously established for HI antibody in volunteer groups using both artificial (Hobson *et al.*, 1972; Dowdle *et al.* 1973; Hobson, Curry & Beare, 1973) and natural virus challenge (Farnik & Bruj, 1966; Pyhala & Aho, 1975), is widely accepted (Stuart-Harris, 1981), and provides a useful indicator of influenza virus vaccine efficacy. The relationship between HI antibody level and resistance to influenza virus infection is well documented (Meiklejohn *et al.*, 1952; Potter & Oxford, 1979).

MATERIALS AND METHODS

Virus strains

Two virus strains were used as antigens in both HI and SRH tests. Influenza A/USSR/92/77 (H1N1) and WRL 105 (H3N2) viruses were both available as stock strains in our laboratory, the former originally obtained from Dr J. J. Skehel, National Institue for Medical Research, Mill Hill, London, the latter from Dr A. S. Beare, Common Cold Reasearch Centre, Salisbury, Wiltshire.

Both viruses were propagated in the allantois of embryonated hen's eggs, and stock virus pools prepared as described elsewhere (Jennings *et al.* 1976).

Serum pairs

All sera for antibodies to either A/USSR/77 (H1N1) or WRL 105 (H3N2) influenza viruses by H1 and SRH were paired sera, collected immediately prior to, and 3–5 weeks following, the intranasal inoculation of adult volunteers with either an H1N1, or an H3N2 attenuated virus, in various influenza virus vaccine studies. The vaccine trials using attenuated H1N1 virus for artificial challenge have been reported elsewhere (Clarke *et al.* 1983*a*, *b*) the virus used in these studies being clone 144-B (H1N1), a recombinant of influenza A virus strain ts/H2N2 and A/USSR/77 (H1N1), obtained from Dr B. K. Murphy, National Institute of Health, Bethesda, Maryland, U.S.A. The virus was administered intranasally to volunteers in a volume of 0·5 ml. The dosage received by each volunteer was $10^{7\cdot0}$ egg infectious doses (EID_{50}). In total, 209 paired sera were available from these studies.

Paired sera collected before and following challenge of volunteers with WRL 105 were obtained from two sources. An influenza virus vaccine study carried out at the University of Sheffield in 1976 provided 25 serum pairs from student volunteers challenged with WRL 105 virus during a dose–response study on an inactivated influenza virus vaccine (Potter *et al.* 1977). The remaining 45 serum pairs, collected from individuals prior to and following the intranasal inoculation of WRL 105 virus during vaccine trials, were kindly supplied by Dr G. Appleyard, Wellcome Research Laboratories, Beckenham, Kent. In both studies, WRL 105 virus (H3N2), an attenuated recombinant of A/Finland/4/74 (H3N2) and A/Okuda/57 (H2N2), bearing the surface antigens of the A/Finland virus, had been given intranasally to volunteers in 0·5 ml volumes containing $10^{7\cdot0}$ EID_{50}.

Since their collection, all serum pairs had been stored at −20 °C. For the present study, all were tested as paired sera by HI and SRH, over a 12-month period.

Serological tests

(*a*) *Haemagglutination-inhibition (HI) tests*

HI tests on paired sera were carried out using standard procedures and a microtitre method (Jennings *et al.* 1981; Jennings *et al.* 1984). Both A/USSR/77 and WRL 105 virus antigens were used at eight haemagglutinating units. All sera were pre-treated with receptor-destroying enzyme, RDE (Burroughs-Wellcome Ltd, Beckenham, Kent), by overnight incubation at 37 °C to remove non-specific inhibitors, and subsequently heated at 56 °C for 30 min. A four fold or greater rise in HI antibody titre in post-challenge sera was considered significant and taken to indicate infection (Stuart-Harris & Schild, 1976).

(*b*) *Single radial haemolysis (SRH) tests.*

The procedures used for SRH tests on paired sera have also been described elsewhere (Goodeve, Jennings & Potter, 1983; Jennings *et al.* 1984). Essentially the gels for haemolysis were prepared using the methods of Oxford, Yetts & Schild (1982), except that veronal buffered saline (VBS) was used as diluent throughout (Kurtz *et al.* 1980). Sheep erythrocytes were sensitized using 30000 haemagglutinating units (HAU) of A/USSR/77, or 20000 HAU of WRL 105 per ml of erythrocytes. Wells of 2 mm diameter were cut into the prepared gels contained in 10×10 mm^2 petri dishes, and each well filled with 5 μl of test or control serum inactivated at 56 °C for 30 min, using a Drummond microdispenser (Drummond Scientific Company, Broomhall, Pennsylvania, U.S.A.). The diameters of the haemolytic zones observed, including the well, were measured using a Transidyne Calibrating Viewer (Transidyne General Corporation, Ann Arbor, Michigan, U.S.A), and zone areas of haemolysis calculated.

Statistical methods

The χ^2-test was used for determination of significant differences between HI and SRH tests on paired sera.

RESULTS

Standardization of the SRH test

The SRH test was standardized with respect to both intraplate and interplate variation, for both H1N1 and H3N2 viruses. Intraplate variation was assessed using five human sera with varying antibody levels to A/USSR/77 virus (H1N1) and five, different sera having varying antibody levels to WRL 105 (H3N2) virus. Each serum was tested nine times in replicate on a single plate, to determine the extent of zone area variation. The results showed that for A/USSR the differences between the smallest and the largest zone area, for five sera tested, ranged from 6·8 % to 47·1 %. The corresponding figures for sera with antibodies to WRL 105 were 10·8 % and 41·6 %. The mean difference in zone area variations, for all five sera tested against A/USSR/77 virus, was 22·7 % and this value, plus two standard deviations, equivalent to an increase in 53·9 % in zone area, taken as indicative of a significant difference in antibody levels between paired sera tested against A/USSR/77 on the same immunoplate. For WRL 105 virus similar calculations gave a mean difference of 18·9 % for the five sera tested, and a significant percentage

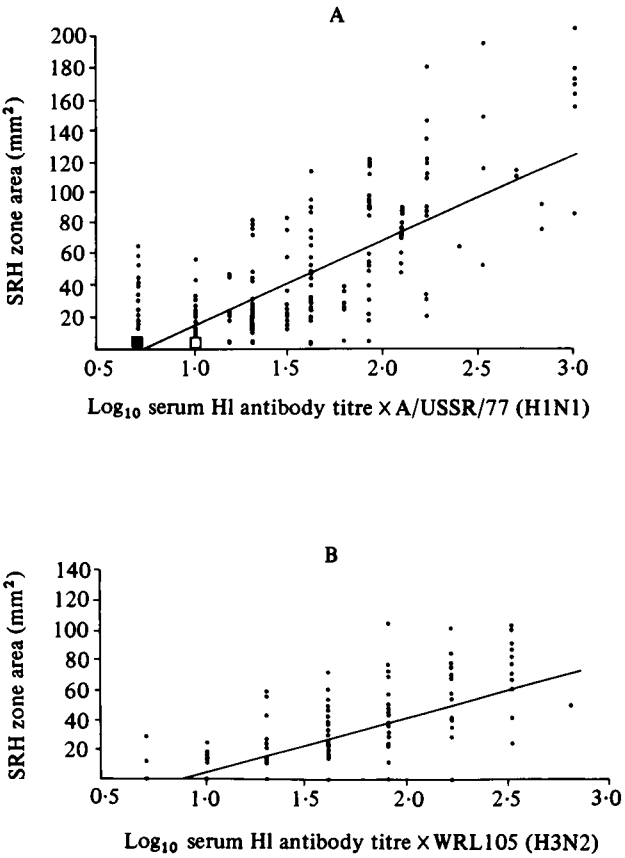

Fig. 1. Scattergrams showing the correlation between HI and SRH antibody levels in sera from volunteers. (A) A/USSR/77 (H1N1) virus: 356 sera. Correlation coefficient, $r = 0{\cdot}78$; $P = < 0{\cdot}01$. ■, 98 samples; □, 61 samples. (B) WRL 105 (H3N2) virus: 140 sera. Correlation coefficient, $r = 0{\cdot}69$; $P = < 0{\cdot}01$.

increase in SRH zone area between paired sera tested on the same immunoplate, to be 44·5 %. Thus, a figure of $\geqslant 50$ % increase in zone area was taken to represent a significant rise in SRH antibody titre against both virus strains.

Interplate variation was eliminated by inclusion of a positive standard serum for each virus on each plate and the zone area readings obtained with these used to normalise the results.

Correlation between HI and SRH tests

(*a*) *For individual sera*

Correlation between HI and SRH tests for both A/USSR/77 and WRL 105 antibodies in individual sera were assessed using scattergrams. Fig. 1 A shows an excellent correlation between the two tests when the results from 356 sera tested against A/USSR were analysed. Good correlation was also obtained for 140 sera tested against WRL 105 by both tests (Fig. 1 B).

Table 1. *Comparison of HI and SRH tests for determining the antibody responses to H1N1 and H3N2 viruses in serum pairs*

Virus antigen	No. of serum pairs tested	Number (%) serum pairs showing: Similar responses by HI and SRH: Total	Antibody rises by both tests	Antibody levels unchanged in both tests	Dissimilar responses by HI and SRH: Total	Antibody rise by HI: unchanged SRH	Antibody rise by SRH unchanged HI	Correlation* of HI and SRH antibody responses
A/USSR/77 (H1N1)	203	164 (80·7)	14 (6·9)	150 (73·9)	39 (19·2)	26 (12·8)	13 (6·4)	Significant ($\chi^2 = 3{\cdot}88$), $p = < 0{\cdot}05$
WRL 105 H3N2	70	54 (77·1)	26 (37·1)	28 (40·0)	16 (22·9)	7 (10·0)	9 (12·9)	Not significant

* Correlation between serum pairs showing similar results by HI and SRH, and serum pairs showing dissimilar results by these tests.

(*b*) *For serum pairs*

The correlation between HI and SRH antibody responses to the A/USSR/77 and WRL 105 viruses were also determined. Of 203 pairs tested using A/USSR/77 only 39 (19·2 %) showed dissimilar responses, i.e. significant rises by one or other of the two tests (Table 1). Of 70 serum pairs tested against WRL 105, 16 (22·9 %) showed dissimilar responses, the remaining 54 (77·1 %) showing either significant increases or no increase by both tests. For serum pairs tested against the H3N2 virus, there was no significant difference between the HI and SRH tests, while for serum pairs tested against A/USSR/77, the difference observed in the antibody responses between the two tests was significant at the 5 % level.

Relationship of pre-challenge serum HI antibody levels to protection against H1N1 or H3N2 infection

H1N1 virus infection

In order to determine the pre-challenge serum HI or SRH antibody levels that correlate with protection against clone 144-B (H1N1) virus infection, serum pairs collected from volunteers prior to and following intranasal inoculation with this virus were tested for HI antibody to A/USSR/77 virus (H1N1). In total, 209 serum pairs were tested by SRH, and 203 of these were also tested by HI. The pre-inoculation sera were then divided into groups according to the level of HI or SRH antibody present (Table 2). Six groups were formed on the basis of their HI antibody levels and four on the basis of their SRH antibody levels. The incidence of significant antibody responses seen following clone 144-B challenge infection in each of these groups was then determined. The results show that for both HI and SRH test, using A/USSR/77 virus as antigen, there is a reduced incidence of significant antibody responses as pre-challenge serum antibody titres increase. Thus, of 57 individuals whose sera showed HI titres of < 10 prior to inoculation with clone 144-B, 25 (43·8 %) were infected by this virus as evidenced by $\geqslant$ four fold increases in HI antibody titre subsequent to clone 144-B inoculation. In contrast, none of 31 individuals with serum HI antibody titres $\geqslant 320$ prior to clone 144-B inoculation, were found to have been infected. Similar results were obtained, using SRH as a measure of antibody levels and responses (Table 2). Of 36 volunteers with pre-challenge serum SRH antibody levels of < 10 mm^2 in zone area, 10 (27·7 %) showed $\geqslant 50$ % increases in zone area in their post-inoculation sera. Of 76 individuals with SRH antibody levels measuring $\geqslant 30$ mm^2 zone area (average 77·2 mm^2) prior to intranasal installation of clone 144-B, none showed significant increases in SRH zone area in post-inoculation sera. For both tests, an intermediate preinoculation antibody level resulted in an intermediate incidence of infection.

H3N2 virus infection

Although considerably fewer numbers of serum pairs were available for study with respect to the H3N2 strain, WRL 105, an essentially similar pattern of results was observed when significant antibody increases to this virus were correlated with pre-challenge serum antibody levels (Table 2). Thus irrespective of whether HI or SRH was the test employed for determination of antibody levels, there was a close relationship between pre-challenge serum titre and the incidence of infection by

Table 2. *Relationship of pre-challenge serum HI or SRH antibody levels to protection against H1N1 or H3N2 infection*

	Haemagglutination-inhibition (HI)			Single radial haemolysis (SRH)		
Virus antigen	Reciprocal pre-challenge HI antibody titre	No. of volunteers per group	No. (%) of volunteers infected*	Pre-challenge SRH antibody titre	No. of volunteers per group	No. (%) of volunteers infected†
A/USSR/77 (H1N1)	< 10	57	25 (43·8)	< 10	36	10 (27·7)
	10–20	27	7 (25·9)	10–20	51	10 (19·6)
	30–40	27	8 (29·6)	—	—	—
	60–80	29	2 (6·9)	20–30	46	7 (15·2)
	120–240	32	1 (3·1)	⩾ 30	76	0 (—)
	⩾ 320	31	0 (—)	—	—	—
	Total	203	43 (21·2)	Total	209	27 (12·9)
WRL 105 (H3N2)	< 10	11	9 (81·8)	< 10	35	24 (68·6)
	10	20	11 55·0)	10–20	14	6 (42·9)
	20	14	5 (35·7)	—	—	—
	40–80	18	5 (27·7)	20–40	12	4(33·0)
	160–320	7	1 (14·3)	40–80	9	1 (11·1)
	Total	70	31 (44·3)	Total	70	35 (50·0)

* Determined by > fourfold serum HI antibody responses post-challenge.

† Determined by > 50 % increase in zone area in sera collected post-challenge.

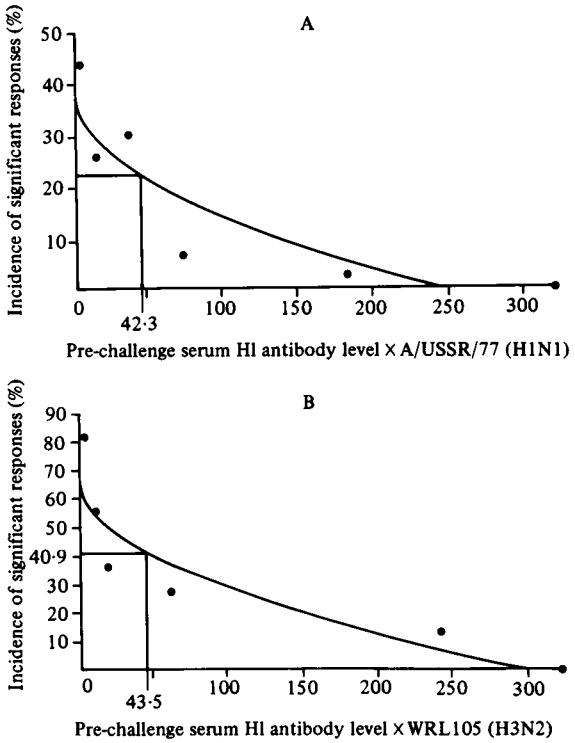

Fig. 2. Relationship of pre-existing serum HI antibody to incidence of infection and determination of PL_{50} values of antibody. (A) A/USSR/77 (H1N1) virus. Extrapolation from the 50% incidence of infection shows the PL_{50} of the HI antibody to be 1:42·3. (B) WRL 105 (H3N2) virus. Extrapolation from the 50% incidence of infection shows the PL_{50} of HI antibody to be 1:43·5.

WRL 105, as shown by $\geqslant$ four fold HI antibody increases, or $\geqslant$ 50% SRH zone area increases subsequent to virus inoculation. Lack of detectable antibody by either test allowed infection by WRL 105 virus to occur, while increasingly higher pre-challenge serum antibody levels progressively prevented infection.

Determination of protective levels of serum HI or SRH antibody against H1N1 or H3N2 infections

To define the levels of serum HI or SRH antibody that will provide a degree of protection against clone 144-B (H1N1) or WRL 105 (H3N2) infection, plots of pre-challenge H1N1 or H3N2, HI or SRH antibody titres against significant antibody responses seen following inoculation of clone 144-B or WRL 105 viruses, were made. Fig. 2 shows the curves obtained when pre-inoculation serum titres were plotted against the incidence of significant antibody responses to clone 144-B (A), or WRL 105 (B). In the populations tested, 43·8% of those with A/USSR/77 serum antibody levels undetectable by HI test showed significant antibody responses to clone 144-B, while 81·8% of individuals with no detectable HI antibodies to WRL 105 showed significant antibody increases. If these values are taken to indicate the maximum incidence of infection that could be achieved by

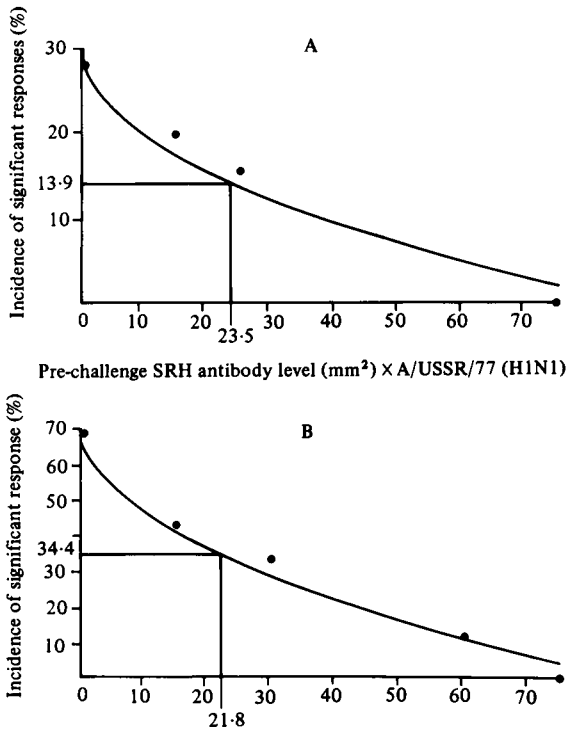

Fig. 3. Relationship of pre-existing serum SRH antibody to incidence of infection and determination of PL_{50} values of antibody. (A) A/USSR/77 (H1N1) virus. Extrapolation from the 50% incidence of infection shows the PL_{50} of SRH antibody to be a zone area of 23·5 mm². (B) WRL 105 (H3N2). Extrapolation from the 50% incidence of infection shows the PL_{50} of SRH antibody to be a zone area of 21·8 mm².

these virus strains in these populations, then 21·9% for clone 144-B and 40·9% for WRL 105, represent, respectively, the 50% infective capabilities of these strains in these populations. By extrapolation, the corresponding levels of serum HI antibody which will protect 50% of the respective populations against infection with clone 144-B or WRL 105, can be determined. For clone 144-B, the 50% protective level (PL_{50}) of serum antibody is a titre of 42·3, for WRL 105, the value is 43·5 (Fig. 2).

Plots can be drawn, and similar extrapolations carried out to determine PL_{50} values of serum antibody, against clone 144-B or WRL 105 viruses, using the SRH test to measure antibody levels and responses. Fig.3A shows that when measured by SRH, the incidence of significant antibody responses in those individuals lacking detectable antibody to A/USSR/77 prior to clone 144-B challenge, was 27·7%; for WRL 105 (Fig. 3B), the equivalent value is 68·8%. Thus, the 50% infective capabilities of these viruses in these populations, assessed using the SRH test, are 13·9 and 34·4% respectively. Extrapolation yields PL_{50} values of serum SRH antibody of 23·5 mm² zone area for clone 144-B (H1N1) and 21·8 mm² for WRL 105 (H3N2).

DISCUSSION

Serum antibody is generally regarded as the closest, readily measurable correlate with immunity against influenza infection in man (Meiklejohn *et al.* 1952; Potter & Oxford, 1979). The assay for the measurement of HI antibody was standardized as long ago as 1953 (W.H.O. Expert Committee on Influenza, 1953) and although the concept of a level of circulating HI antibody that could be equated with protection against influenza was first proposed at about the same time (Meiklejohn *et al.* 1952), it was not until 20 years later that a serious attempt was made to define such a value (Hobson *et al.* 1972). These workers (Curry & Beare, 1972; Hobson *et al.* 1973), determined the results of challenge infection with attenuated virus strains in approximately 1000 volunteers of known pre-inoculation serum HI antibody titre, found an inverse relationship between the pre-challenge antibody titre and the likelihood of infection, and defined the 50 % protective antibody level against A and B influenza viruses as being a serum HI titre of 18–36. It is now generally accepted that a serum HI antibody titre of 30–40 represents a protective level of antibody against influenza A viruses (Potter & Oxford, 1979). Several studies in which the incidence of natural infection has been determined in populations of known pre-existing serum HI antibody titres, have been in general agreement with these values (Farnik & Bruj, 1966; Dowdle *et al.* 1973; Pyhala & Aho, 1975; Delem & Jovanovic, 1978). In the present studies, using attenuated H1N1 and H3N2 influenza A viruses for artificial challenge of volunteers, serum HI antibody titres of approximately 40 were calculated to represent 50 % protective levels (PL_{50}) against both virus strains.

In recent years, SRH has been extensively employed for the measurement of antibody against both A and B viruses (Vaananen *et al.* 1976; Delem & Jovanovic, 1978; Chakraverty, 1980; Goodeve *et al.* 1983) and the test correlates well with the HI for the measurement of serum antibodies to influenza A viruses (Russell *et al.* 1975; Schild *et al.* 1975; Farrohi *et al.* 1977; Jennings *et al.* 1984). In the present studies the two tests showed good correlation for the measurement of both H1 and H3 antibodies.

SRH is a more economical, rapid and reliable test for the measurement of serum antibodies against influenza A viruses in our hands, than the conventional HI technique. For influenza B viruses the HI test is an unreliable indicator of immunity, and is of low sensitivity (La Montagne, 1980; Wright, Bryant & Karzon, 1980), and SRH appears superior for the measurement of antibodies to this virus (Chakraverty, 1980; Oxford, Yetts & Schild, 1982; Goodeve, Jennings & Potter, 1983; Mancini *et al.* 1983). For these reasons, SRH should replace the conventional HI test for routine laboratory measurement of such antibodies. It is therefore of importance to determine the SRH antibody titre that correlate with protection against influenza virus infection, as such titres provide valuable yardsticks in the assessment of influenza virus vaccine efficacy. Earlier studies from this laboratory have indicated that a zone area of 45 mm^2 represents the PL_{50} of SRH antibody against challenge with an attenuated strain of influenza B (Goodeve *et al.* 1983).

In the present studies, for both H1N1 and H3N2 influenza viruses, a zone area of 20–25 mm^2, represents the PL_{50} of SRH antibody and although artificial challenge with attenuated virus strains was employed, this figure is in agreement

with that obtained by other workers (Delem & Jovanovic, 1978) during an outbreak of wild-type H3N2 influenza virus infection. However, further volunteer studies with other virus strains and using virus isolation as a measure of infection, as well as determination of the SRH antibody levels correlating with protection against wild-type strains in natural influenza virus outbreaks are necessary to confirm these results.

These findings, together with those of a previous study with influenza B (Goodeve *et al.* 1983), provide parameters enabling the SRH test to be used more effectively for the measurement and interpretation of antibody levels to influenza viruses.

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