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Use of single radial immunodiffusion test for serological studies in volunteers inoculated with live attenuated influenza virus

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SUMMARY

Pre- and post-vaccination serum samples from 278 volunteers, who were given live influenza vaccines, were tested by haemagglutination inhibition (HI) and single radial immunodiffusion tests(SRDT) for antibody to influenza A/Hong Kong/1/68 (H3N2) virus envelope antigens. Those with high antibody titres detected in both tests were less frequently infected, and 85% of the 159 infected showed rises by HI and 70% by SRDT. Similarly, 69 pairs were tested for antibody to Hong Kong (N2) neuraminidase by neuraminidase inhibition test (NI) and by SRD tests. Those with high titres in both tests resisted infection and those who were infected showed a rise in antibody detected both by NI and SRD tests. In general, SRDT was less sensitive than HI and NI in detecting antibody and antibody rises, but in some volunteers it did detect antibody rises which were not detected by conventional tests. Because of its simplicity and speed it appeared to be of use in evaluating such vaccines.

INTRODUCTION

The measurement of circulating antibodies against antigens of the influenza virus is important in the study of influenza epidemiology and in the assessment of the efficacy of vaccines. Antibodies directed against the envelope proteins of haemagglutinin and neuraminidase are conventionally measured using some version of the haemagglutination-inhibition (HI) test (Hirst, 1942) and the neuraminidase-inhibition (NI) test (Webster & Laver, 1967). Complement-fixation tests (CFT) are used to detect antibody to the internal ribonucleoprotein antigen. The technique of single radial immunodiffusion has been applied to measurement of antibodies and antigens (Mancini, Carbonara & Heremans, 1965; Vergani, Stabilini & Agostini, 1967). Schild, Henry-Aymard & Pereira (1972) described the use of a single radial immunodiffusion test (SRDT) for influenza antibodies. The test is more convenient and rapid than the HI or NI tests or CFT (Schild *et al.* 1972). In the present study the SRDT is compared with the conventional tests in the

study of sera from volunteers inoculated with live influenza virus. The methods are compared for sensitivity in detecting initial serum antibody levels, in predicting succeptibility to infection and in detecting antibody rises after infection.

MATERIALS AND METHODS

Volunteers

The sera studied were from 278 volunteers who had taken part in a number of trials at the Common Cold Unit, Harvard Hospital, Salisbury, between 1968 and 1970. Standard methods of isolation of volunteers, nasal inoculation of virus, the taking of nasal washings and clinical observation were used (Tyrrell, 1963; Beare, Bynoe & Tyrrell, 1968).

Inoculation of volunteers

After the initial blood sample had been taken, each volunteer was given live influenza virus by intranasal inoculation. The virus used in each case was influenza A/Hong Kong/1/68 (H3N2) or a recombinant having the same surface antigens (H3N2). In most trials the dose used was $10^{5\cdot5}$ EID 50. In some $10^{6\cdot5}$ EID 50 was used. Nasal washings were taken on the 3rd and 4th days after inoculation and virus isolation from washings was attempted.

Serology

Second serum samples were obtained from volunteers 2–3 weeks after inoculation. All sera were stored at $-20°$ C. HI tests (Tyrrell, Peto & King, 1967) were carried out on all of the 278 pairs of sera, and NI tests (Aymard-Henry *et al.* 1973) on 69 pairs. All were tested by single radial diffusion in plates containing X31 virus (Kilbourne, 1969) containing surface antigens (H3 and N2) identical with those of A/Hong Kong/1/68 virus. Those sera on which the NI test was performed were also tested by radial diffusion in plates containing X15 HK virus (surface antigens HEq1N2).

Single radial diffusion test

The immunoplates employed (Schild *et al.* 1972) consist of agarose gel incorporating a homogeneous suspension of intact, purified influenza virus. Test sera (5 μl. volumes) are placed in 2 mm. diameter wells cut in the agar, and the presence of antibody to viral surface antigens is detected by the appearance of a zone of opalescence surrounding the wells (see Plate 1).

In plates containing A/Hong Kong/1/68 (H3N2) virus (X31 strain), opalescence may be due to either anti-haemagglutinin or anti-neuraminidase or both. In plates containing the recombinant strain A/Equi1/Prague/56(HEq1)–A/Hong Kong/1/68 (N2) only anti-neuraminidase antibody is detected as anti-HEq1 antibodies are not found in human sera.

In this study the size of reaction is recorded as the diameter of the opalescent zone. The minimum zone diameter considered as significant was taken as 2·2 mm. A significant difference between two readings is one of 10% or greater (Schild, Berryman, Pereira & Henry-Aymard, 1973).

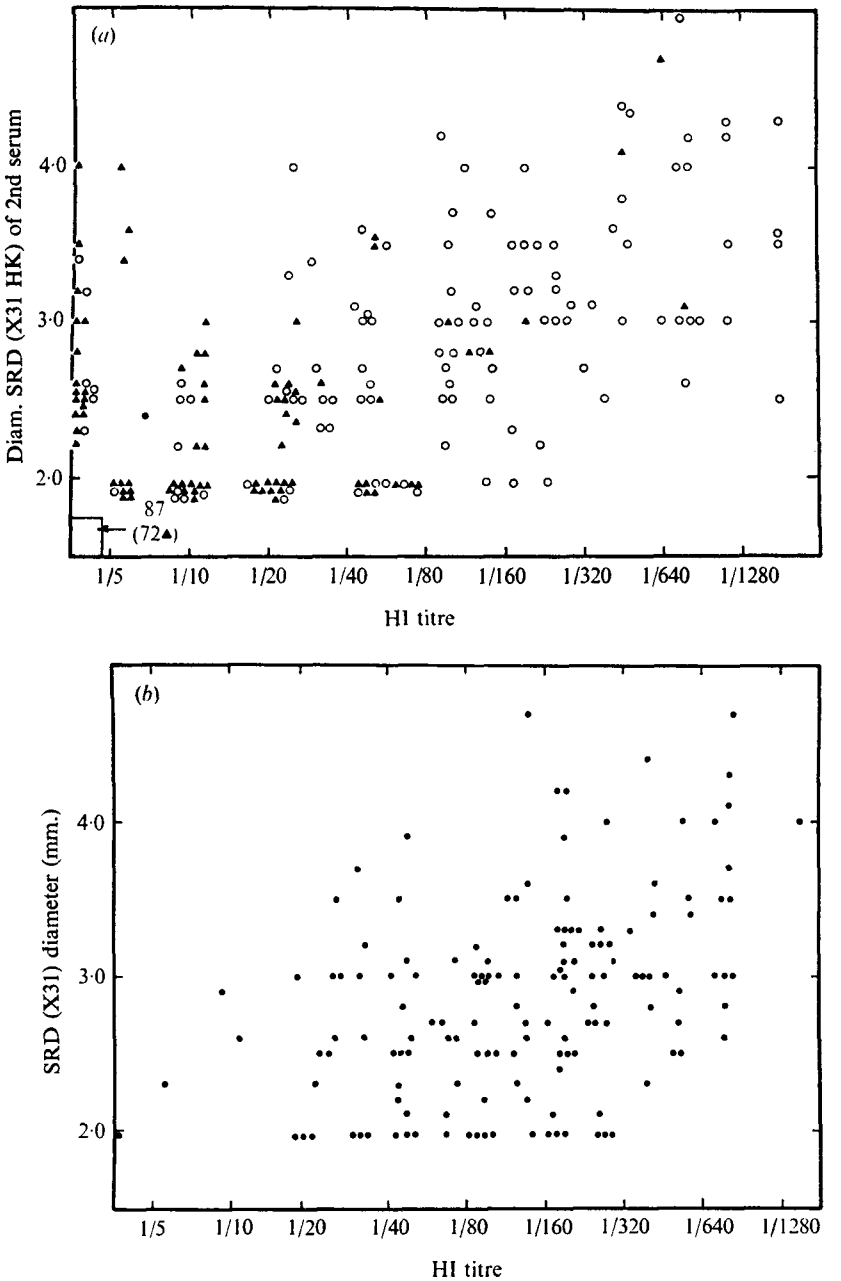

Fig. 1. (*a*) The results of titrating the first specimens of sera from subjects who did and did not become infected when subsequently inoculated with live influenza vaccine. Both HI and SRD tests were done with viruses of the Hong Kong (H3N2) serotype and the challenge viruses belonged to this serotype also. There is a correlation between the results of the two tests ($r = 0{\cdot}6437$, $n = 277$, $P < 0{\cdot}001$). ▲, Subjects who became infected: ○, subjects who did not become infected. (*b*) The results of titrating the second specimens of sera from subjects who became infected. There is again a positive correlation (for all second sera $r = 0{\cdot}5128$, $n = 276$, $P < 0{\cdot}001$).

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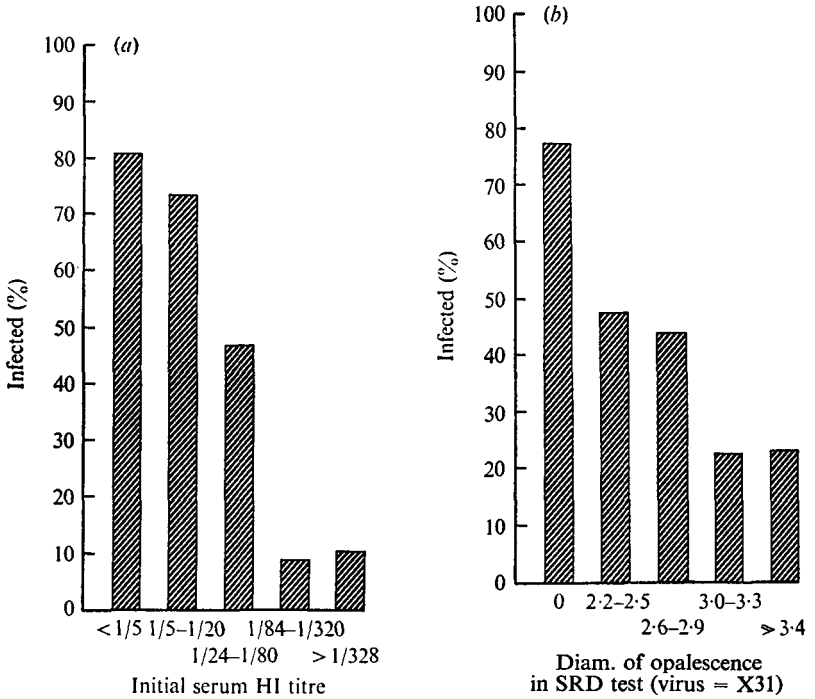

Fig. 2. Percentage of volunteers who were infected in relation to their serum antibodies before infection: (*a*) as measured by HI and (*b*) by SRD.

Evidence for infection of volunteers

For the purposes of this study, a subject was judged to have been infected by the inoculated virus if one or both of the following criteria were fulfilled: (*a*) virus isolated from nasal washings on 3rd and 4th days; (*b*) a fourfold or greater rise in HI titre 2–3 weeks after inoculation.

RESULTS

Antibody measurement

The correlation between HI titres and the diameter of the opalescent zone on X31 plates is shown in Fig. 1(*a*) and (*b*). Fig. 1(*a*) shows the results for initial (pre-inoculation) sera. There is a positive correlation between the results obtained by the two tests; but of the 277 sera, there were 49 (18 %) in which antibody was detected by the HI test and not by SRD and 22 (8 %) in which SRDT but not HI indicated the presence of antibody. Fig. 1(*b*) represents post-vaccination sera: only sera from those volunteers who were infected by the inoculated virus, as judged by the above criteria, were included. Again a positive correlation was found. In 21 of the 152 sera (13·8 %) no antibody was detected by SRDT.

Prediction of susceptibility

In Fig. 1(*a*), which shows all initial sera tested by the two methods, those subjects who were infected by the inoculated live virus are represented by filled

Table 1. *Rising antibody titres detected by HI and SRDT, and rate of virus isolation from the respiratory tract*

No. of paired sera with single radial diffusion (virus = X31) showing	No. of paired sera with HI titres showing: No rise	Significant rise	Totals
No rise	129 (12:9 %)*	36 (18:50 %)	165 (30:18 %)
Significant rise	12 (4:33 %)	99 (75:76 %)	111 (79:71 %)
Totals	141 (16:11 %)	135 (93:69 %)	276 (109:39 %)

* Figures in parentheses show the number and the percentage of subjects in each group yielding virus from nasal washings on day 3 or 4 after inoculation.

triangles; those who did not become infected, though inoculated with live virus, are represented as open circles. It is clear that the greatest incidence of infection is amongst those with low antibody measured by either test. There are some subjects who succumb to infection despite high initial antibody levels, whether measured by the HI or SRDT. The proportion of 'susceptible' people in groups with various initial antibody titres is shown in Fig. 2 (*a*) and (*b*). For both tests, the proportion of subjects becoming infected decreases with increasing initial antibody titre.

Antibody responses to infection

Table 1 represents the changes in antibody titres in all pairs of sera as measured by HI and by SRDT using X31 immunoplates. Figures in parentheses indicate the number of subjects in each category from whom virus was isolated in nasal washings taken on the 3rd or 4th day after inoculation. In the majority of subjects, virus isolation is accompanied by a significant rise of antibody measured by both methods. There were 12 who were infected, as judged by virus isolation, in whom no serological evidence for infection appeared by either test. In a further 36 there was a fourfold or greater rise in HI titre with no rise shown in the SRDT. In 12 (4 of whom yielded virus from nasal washings) a rise of antibody titre appeared in the SRDT with no change in HI titre. If the criteria of infection are enlarged to include (*a*) virus isolation, (*b*) fourfold or greater rise of HI titre and (*c*) significant rise on SRDT, then from Table 1, the number infected was

$$12 + 36 + 12 + 99 = 159.$$

Of these, 135 (85 %) would have been revealed by the HI test alone and 111 (70 %) by the SRTD alone, using X31 plates.

Antibody changes in a random sample of the paired sera tested are shown graphically in Fig. 3. Most pairs are represented as an oblique line, indicating a rise of antibody titre by both tests. For some the line is vertical, indicating a rise in HI titre with no change in SRD. Three pairs are represented by a horizontal line, in which there is an increase in the SRDT but no change of HI titre. Two of these pairs also showed an increase on the X15 HK plate, suggesting that the antibody rise may be due to an increase in anti-neuraminidase antibody

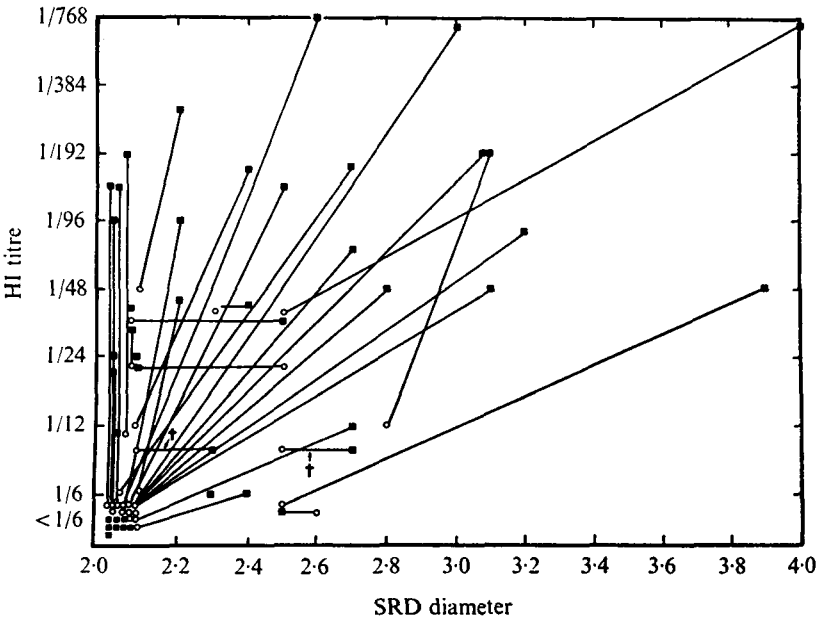

Fig. 3. Antibodies of volunteers before and after inoculation as measured against Hong Kong by HI and SRD. The first and second serum values of each subject are joined by a line. In those indicated thus† a rise of antineuraminidase antibody was detected by SRDT using X15. ○, Before vaccination; ■, after vaccination.

Table 2. *Rising antibody titres detected by NI and SRDT, and rate of virus isolation from the respiratory tract*

No. of paired sera with single radial diffusion (virus = X15HK) showing	No. of paired sera with NI titres showing: No rise	Significant rise	Totals
No rise	28 (8:29%)	10 (7:70%)	38 (15:40%)
Significant rise	8 (7:87%)	23 (20:87%)	31 (27:87%)
Totals	36 (15:42%)	33 (27:82%)	69 (42:61%)

* Figures in parentheses show the number and the percentage of subjects in each group yielding virus from nasal washings on day 3 or 4 after inoculation.

only: this would be measured as a rise on the X31 plate, but might not be detected by HI tests.

Anti-neuraminidase antibody

The neuraminidase inhibition (NI) test was carried out on a random selection of 69 pairs of the total number of paired sera available. These 69 pairs of sera were also tested by SRD in plates containing the virus X15 HK(HEq1N2), to detect anti-neuraminidase antibodies. The correlation between NI and SRDT is shown in Fig. 4(*a*) and (*b*). The correlation is better for the post-infection sera; in both cases there are more sera in which antibody is detected by NI test alone than by SRDT alone. In Table 2 it can be seen that in most subjects who were

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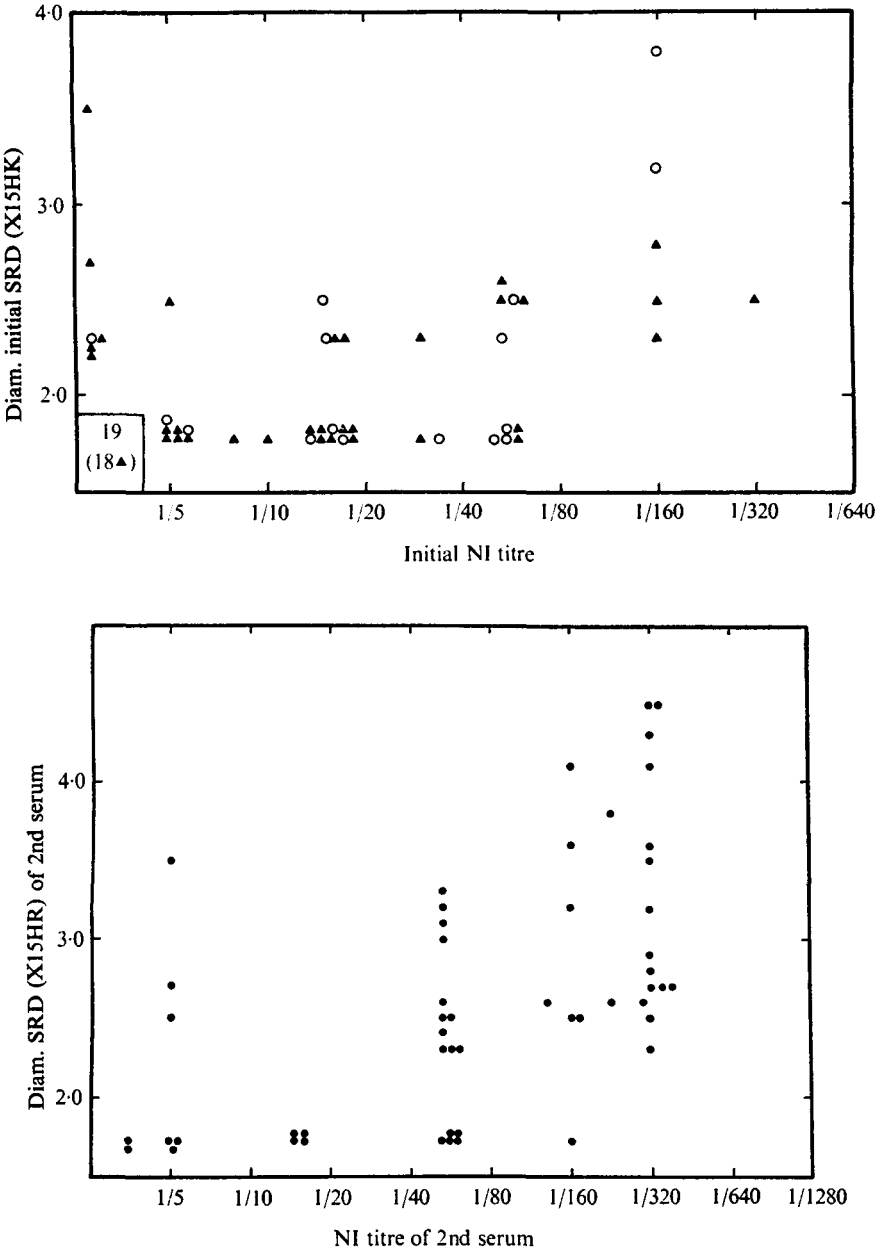

Fig. 4. (*a*) The results of titrating the first specimens of serum from subjects who did and did not become infected when subsequently inoculated with live influenza vaccine. NI and SRD tests were done with the N2 serotype. ▲, Infected; ○, Uninfected. (*b*) The results of titrating the second specimens of serum from subjects who became infected.

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infected as judged by virus isolation, there is a rise of anti-neuraminidase antibody. In some of these the rise is detected only by NI test, and in a similar number by SRDT only. In a few there is no rise in either test.

DISCUSSION

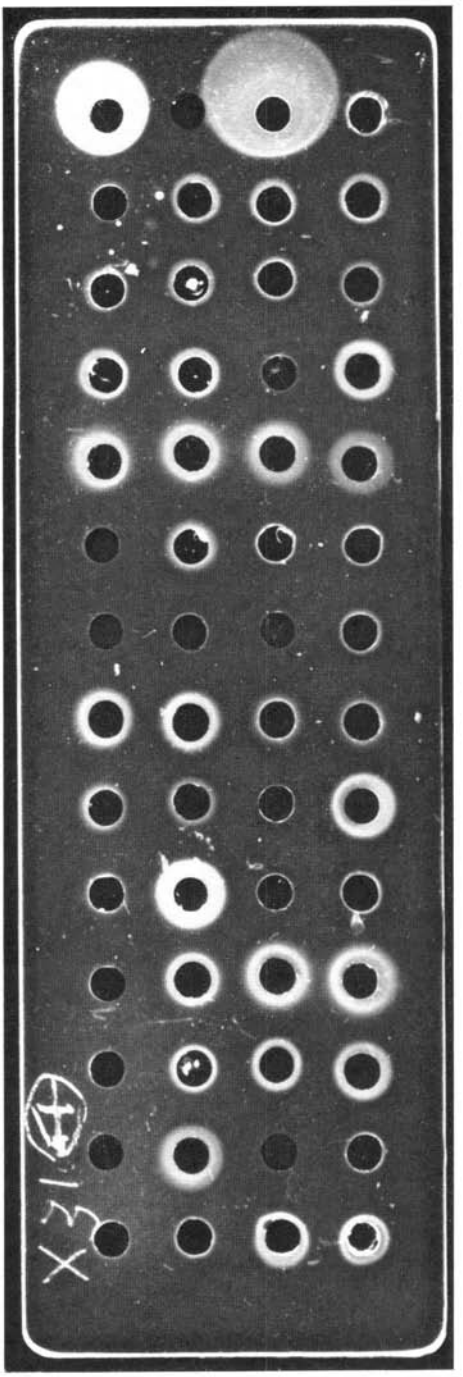
As live influenza vaccines are developed serological studies on large numbers of people will be necessary for their evaluation and simple and precise methods are therefore desirable. The single radial diffusion test has several advantages over conventional tests in serological studies for this purpose (Schild *et al.* 1973). It is quick and easy to perform. If necessary, large numbers of sera can be tested in field conditions, and plates can be posted for subsequent reading. Fifty-six sera can be tested on a single plate measuring 1 in. × 3 in. Whole fresh blood may be used if necessary; very small volumes are sufficient, so that finger-prick may be used instead of venepuncture. Since non-specific inhibitors of haemagglutination do not react in SRDT no pretreatment of samples is needed. Plates can be photographed to provide a permanent record. Antibodies against various constituents of the influenza virus can be measured if appropriate recombinant or disrupted virus is incorporated in the agarose gel.

A disadvantage of the method is that greater quantities of virus are needed for the test plates than would be needed for HI tests on the same number of sera, but this problem is minimized by the availability of high yielding recombinant strains of influenza such as X31. As a method of detecting anti-neuraminidase the method offers considerable advantages because of the complexity of the enzyme-inhibition test.

The results of this study of the sera of volunteers given live virus vaccines indicate that SRDT give information which is similar to that which can be obtained from HI and NI tests, although the SRDT have been less sensitive. There is a lower rate of detection of antibody in initial serum samples, and of antibody rises in paired sera when the SRDT is used. The correlation between antibody level and resistance to infection is better when antibody is measured by the HI test than by SRD (Fig. 3*a*, *b*).

The greatest amount of information is obtained when both conventional tests and SRDT are used. However, the SRDT alone provide information on antibody status, susceptibility and serological response which could be of considerable value in the study of live vaccines. The disadvantages of the apparent lower sensitivity of the test must be weighed against the advantages of simplicity and speed in the assessment of large numbers of sera. The sensitivity would be less important in comparisons between different vaccines, and S. R. Mostow, G. C. Schild and W. R. Dowdle (unpublished) have used the test to evaluate the antibody responses following killed influenza vaccines and natural infection and have found the SRDT method to be more sensitive than conventional HI and CF tests in detecting antibody rises. Recent unpublished studies show that a test using 10 μl. of serum and 3 mm. wells is more sensitive than HI.

The relatively poor correlation (Table 1) between antibody titres measured by



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SRD and the results obtained with the HI test suggest that the two tests may be measuring different antibodies. In this connexion it is relevant that J. L. Virelizier, G. C. Schild, R. Postelthwaite & A. C. Allison (in preparation) have shown that antibodies against different antigen determinants of the haemagglutinin molecule can be detected and identified by SRDT. It seems likely from these studies that SRDT is capable of detecting antibody against a wider range of the antigenic determinants of the haemagglutinin molecule than is the HI test. In particular, a modification of the SRDT, involving antibody adsorption procedures, enabled the independent assay of antibody to strain-specific antigenic determinants of the haemagglutinin and cross-reactive determinants (shared between members of a single haemagglutinin subtype) and evidence was obtained suggesting that strain-specific antibody is likely to be more closely related to immunity than cross-reactive antibody. It will be of interest to continue our studies on antibody responses to various types of influenza vaccines using strain-specific and cross-reactive antibody assays as measures of the immune response.

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EXPLANATION OF PLATE

A typical plate showing the zones of opalescence, the diameters of which were measured.