THE TITRATION OF INFLUENZA VIRUS-NEUTRALIZING ANTIBODIES

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(With 2 Figures in the Text)

INTRODUCTION

A new method of cultivating influenza virus in fragments of chick chorio-allantoic membrane has already been described (Fulton & Armitage, 1951). In that paper the method was developed for the assay of virus, and the way virus-neutralizing antibodies could also be measured was indicated briefly. The assay of virus-neutralizing antibodies is described fully in the present paper, with the application of the technique to the differentiation of virus strains.

APPARATUS AND MATERIALS

Familiarity with the basic technique described in the earlier paper will be assumed, and only the few innovations made in it will be discussed.

(i) *Plastic tray*

In washing the tray the following practice has proved effective. After use the tray is flooded with 1·0% hydrochloric acid, and the erythrocytes are carefully dislodged from the bottoms of the cups; the tray is left to soak in the acid for 1 hr. The rubber washer is now removed, washed and dried separately. The tray is washed in running water, the cups being thoroughly cleaned by directing a jet of water into each. The cups are then filled with 10% hydrochloric acid, care being taken that none of the strong acid comes into contact with the steel bolts. After half an hour the tray is again washed in running water and finally rinsed in distilled water. Usually the tray is left to dry inverted in an incubator at 37° C.; if more rapid drying is desired the tray is placed in an oven maintained at 50° C. When dry the tray is fitted with its rubber washer, sterilized by irradiation with ultra-violet light and stored in a sterile tin.

(ii) *Egg white*

Fresh egg white is collected with sterile precautions and one part is mixed with nine parts of physiological saline. Most of the egg white readily goes into solution; after half an hour at room temperature the egg-white solution is decanted and stored at 4° C. It will keep for at least a month. For use as a virus diluent it is further diluted 1/10 in physiological saline.

https://doi.org/10.1017/S0022172400019586 Published online by Cambridge University Press

(iii) *Influenza virus strains*

A	PR 8, WS
A′	Barratt (BAR), Rhodes (RHO)
Swine	Shope 15 (SHO)
B	Lee, Crawley (CRA)

Infected allantoic fluids were prepared by inoculating fertile hens' eggs which had been incubated at 39° C. for 10–11 days with 40 mm.^{3} of a 10^{-4} dilution of seed virus. The fluids were collected after 48 hr. incubation at 36° C., mixed with an equal volume of broth and stored, sealed in glass ampoules, at −70° C.

(iv) *Sera*

Normal ferret sera and sera from ferrets convalescent after intranasal infection with influenza virus were stored, sealed in glass ampoules, at −70° C. The sera were pools from at least two ferrets. Some of the immune ferret sera were obtained from the WHO Influenza Centre, and had been lyophilized. At the start of this work the dried sera were rehydrated and thereafter stored frozen in ampoules at −70° C.

DETAILED DESCRIPTION OF THE TECHNIQUE FOR TITRATION OF VIRUS-NEUTRALIZING ANTIBODIES

It has already been emphasized in the earlier paper that it is impracticable to titrate neutralizing antibodies by adding serum directly to the cups; for sera are strongly inhibitory in a non-specific way, and any specific anti-haemagglutinin in them would mask any haemagglutinin produced, so concealing virus multiplication. The method adopted, therefore, was to expose the pieces of chorio-allantoic membrane to the antiserum-virus mixtures for a standard time, and then to transfer them to fresh cups containing buffered glucosol but no antiserum. If infection had occurred in the primary virus-serum mixture, virus would accumulate in the second cup and could be detected in the usual way by haemagglutination.

At first, the time for which the piece of chorio-allantoic membrane was exposed to the hazard of infection in the virus-serum mixture was 1 hr. This proved quite satisfactory with some ferret sera, but a number of convalescent ferrets, especially after infection with A′ strains, failed to show neutralizing antibodies even though complement-fixing antibodies could easily be found.

In consequence, a study was made of ways of increasing the delicacy of the titration. It had been observed in the assay of influenza virus that even in those cups to which a large virus inoculum had been added, no haemagglutinin could be detected for the first 20–24 hr.; if, therefore, the piece of chorio-allantoic membrane was transferred before this time the second cup could be expected to receive the bulk of the new haemagglutinin. It was found that if, in the titration of neutralizing antibodies, the standard period allowed for infection in the virus-serum mixture was prolonged to 18 hr., all the convalescent ferret sera showed specific neutralizing power. It was further found to be more convenient to run the test for a total of only 48 hr., and not for the 72 hr. preferred in the titration of virus, in which maximum titres are desired.

As the precise method is important if the greatest sensitivity is to be achieved an actual titration will be described in detail.

(i) *Determination of ID 50 of virus (BAR)*

Fifty ml. of modified glucosol are mixed with an equal volume of the phosphate buffer (pH 6·8), and to the mixture is added sufficient aqueous penicillin to give 10 units/ml., and sufficient aqueous streptomycin to give 40 μg./ml. This solution is now distributed in 1·0 ml. amounts to the 100 cups in the plastic tray, the two bottom cups being left empty as usual. A piece of chorio-allantoic membrane from a 14-day-old chick embryo is dropped into each cup in rows 1–5 inclusive. An ampoule of virus-infected allantoic fluid is thawed and 0·1 ml. added to 10 ml. of physiological saline containing approximately 1·0% of egg white. From this initial 10^{-2} dilution, serial ten-fold dilutions to 10^{-7} are made by transferring 1·0 ml. of the previous dilution to 9·0 ml. of the diluent, a fresh pipette being used at each stage. With a dropping pipette, 40 mm.^{3} of the 10^{-7} dilution are added to each cup in row 5, 40 mm.^{3} of the 10^{-6} dilution to each cup in row 4 and so on, the first row receiving the 10^{-3} dilution. The top plate is now screwed into position and the tray is rocked for 18 hr. in an incubator maintained at 36° C. At the end of this period the tray is removed, the top plate is taken off and the two are returned to their respective sterile tins. The pieces of membrane are transferred to the lower fifty cups in the tray, the pieces in the cups of row 5 being transferred to the corresponding cups in row 10, the pieces from row 4 to row 9 and so on. As each piece is transferred it is washed in 1·0 ml. of glucosol. By making use of the plastic tray provided by the WHO Influenza Centre for haemagglutinin titrations, it is easy to wash each piece of membrane in a fresh aliquot of the glucosol. The forceps used in making the transfer are rinsed between each piece by momentarily dipping them in boiling saline.

The tray is reassembled, fitted on to the platform of the rocking motor and returned to the incubator for a further 24 hr.; then the cups in rows 6–10 inclusive are tested for haemagglutinin in the usual way. The result is shown in Table 1 (i). The virus ID 50 is computed by Thompson's moving average method (Thompson, 1947) and is found to be 20×10^{-6} mm.^{3} (= 40 mm.^{3} of a $10^{-6} \times \frac{1}{2}$ virus dilution).

(ii) *Specific serum neutralization*

Thirty-five ml. of modified glucosol are mixed with an equal volume of the phosphate buffer (pH 6·8). To 20 ml. of this diluent are added 0·2 ml. of the BAR ferret antiserum, and the mixture is inactivated by placing in a 56° C. water-bath for half an hour.

From this initial inactivated 10^{-2} dilution of serum, three further dilutions are made in steps of 1/3 by transferring 6·0 ml. to 12 ml. of the diluent, and so on.

Four sterile tubes are set up: tube 1 is filled with 11·0 ml. of the 10^{-2} serum dilution, tube 2 with 11·0 ml. of the $10^{-2} \times 1/3$ dilution, tube 3 with 11·0 ml. of the $10^{-2} \times 1/9$ dilution, and tube 4 with 11·0 ml. of the $10^{-2} \times 1/27$ dilution. To each tube is now added sufficient aqueous penicillin to give 10 units/ml. and sufficient

virus so that each millilitre contains 100 virus ID 50; in this case 0·44 ml. of a $10^{-4} \times \frac{1}{2}$ dilution of the BAR virus.

The serum-virus mixtures are incubated in a water-bath at 37° C. for 1 hr., and then 10 ml. of tube 1 are distributed in 1·0 ml. amounts to the cups of row 1, 10 ml. of tube 2 distributed to the cups of row 2 and so on; a piece of chorio-allantoic membrane from a 14-day-old chick embryo is dropped into each cup in rows 1–4 inclusive. These are referred to as the primary cups.

Table 1. *The titration of influenza virus (BAR) neutralizing antibodies*

(i) Computation of BAR virus ID 50

Initial virus dilution ($\log_{10}$)	Inoculum (mm.³ of seed $\times 10^{-6}$)	Result:* No. of cups +	−
$\bar{3}$	40,000	10	0
$\bar{4}$	4,000	10	0
$\bar{5}$	400	10	0
$\bar{6}$	40	6	4
$\bar{7}$	4	2	8

ID 50 = $\bar{7}\cdot7 = 20 \times 10^{-6}$ mm.³

(ii) Computation of titre of neutralizing power of a homologous ferret antiserum, with 100 virus ID 50

Serum dilution in primary cups ($\log_{10}$)	Result:* No. of cups +	−
$\bar{2}\cdot00$	0	10
$\bar{3}\cdot52$	1	9
$\bar{3}\cdot04$	8	2
$\bar{4}\cdot52$	10	0

Serum titre = $\bar{3}\cdot23$

* + denotes the presence of haemagglutinin.

The cups in rows 7–10 inclusive are filled with 1·0 ml. amounts of the standard diluent containing both penicillin and streptomycin, rows 5 and 6 not being used in this particular experiment.

The tray is assembled and rocked in the incubator for 18 hr., after which time the pieces of membrane in the cups of the upper four rows are transferred, with the precautions already described, to the corresponding cups in the lower four rows. The tray is returned to the incubator and rocked for a further 24 hr., after which the cups in rows 7–10 are tested for haemagglutinin in the usual way.

The result is shown in Table 1 (ii). The titre of the neutralizing power of the serum is defined as the $\log_{10}$ of the serum dilution (final) in the primary cups which will most probably protect from infection with 100 ID 50 of virus half of a large number of replicates. Again using Thompson's moving average method, the titre of this BAR ferret antiserum with homologous virus is found to be $\bar{3}\cdot23$ (= 1/589).

https://doi.org/10.1017/S0022172400019586 Published online by Cambridge University Press

CRITIQUE OF THE TECHNIQUE

It is essential that the preliminary incubation of the virus-serum mixture, before the addition of the piece of chorio-allantoic membrane, should be made in glass tubes as described. It would, of course, have been simpler to have used the plastic tray throughout, but if this is attempted the sensitivity of the neutralization test is greatly reduced. Why this is so is not understood, though it is possible to speculate that adsorption of the serum component is occurring at the Perspex-fluid interface. One hour at 37° C. has been chosen as a convenient time for the preliminary incubation of the virus-serum mixture; it may be that a shorter time would have been sufficient, but no advantage is gained by prolonging the time at a lower temperature because the virus is not stable. The serum is best inactivated after some dilution in the suspending fluid, and for this reason the antibiotic is not included until a later stage. The omission of streptomycin from the primary cups has no particular significance but is convenient, since penicillin is all that is required.

In the assay of influenza virus it had been found possible to use chorio-allantoic membranes from embryos 9–16 days old. In the neutralization test irregular results were sometimes observed if the older embryos were used, and it has seemed wise to restrict the range to 10–14-day embryos.

Washing the pieces of membrane during transfer is a very quick and simple procedure, though from the description it may seem formidable. To ensure uniformity the pieces used for the virus assay have also been washed during transfer, but this is not strictly necessary. It is essential to rock the tray both during the first 18 hr. and again after transfer for the remaining 24 hr.

For the estimation of serum titres it has been thought sufficient to calculate the ID 50 by a simple interpolation method; Armitage & Allen (1950) have found that in many cases Thompson's method gives results well within the range of sampling variation of the probit estimate.

(i) *The use of* 100 *virus ID* 50

The apparent titre of neutralizing antibodies in a serum is related to the number of virus ID 50 used in the test (Fig. 1); this is a necessary consequence of the 'percentage law'. For this reason the virus concentration in the primary cups must, if the test is to be sensitive, be kept at a minimum. Normal sera possess a significant non-specific neutralizing power so that the number of virus ID 50 required will be determined both by the kind of serum being studied and by the lowest dilution at which it is to be used. With ferret sera and confining attention to the neutralizing power of dilutions not less than 1/100, 100 virus ID 50 provide the most sensitive level for the assay of specific neutralization. With this dose of virus mixed with 10^{-2} normal inactivated ferret serum, there is a strong probability that all the pieces of membrane exposed to risk will become infected. Of 120 such replicates, 111 were positive and 9 were negative. Thus the titres recorded in this paper may reasonably be assumed to represent titres of specific neutralizing antibodies.

If more concentrated sera are to be measured a greater virus concentration is required (Fig. 2).

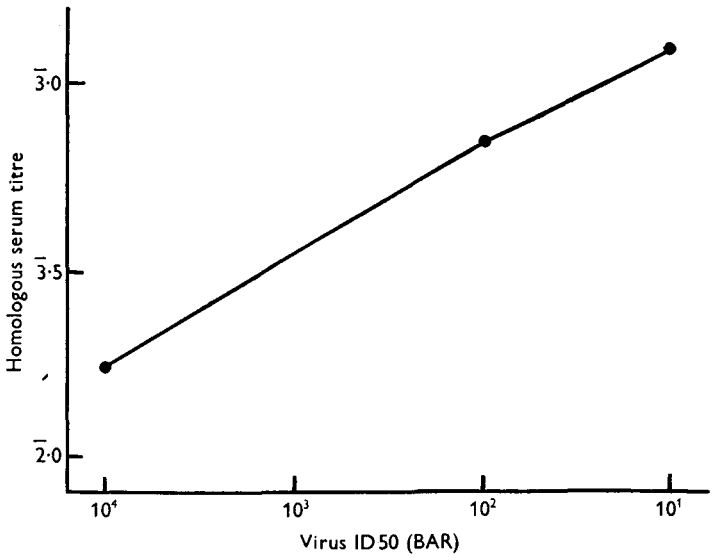

Fig. 1. The relation between the apparent titre of neutralizing antibodies (BAR), and the number of homologous virus ID 50 used in the test

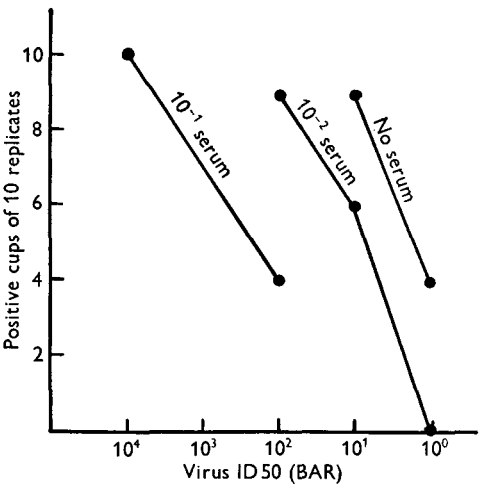

Fig. 2. Non-specific neutralization of influenza virus (BAR) by normal ferret serum

(ii) *Reproducibility of the results*

The same BAR antiserum was titrated on three separate occasions with the same homologous virus pool; later a fresh pool of homologous virus was prepared and the titration was repeated. The ID 50 of the first virus pool was 10×10^{-6} mm.^{3}, and of the second virus pool 20×10^{-6} mm.^{3}. By the first estimate the serum titre was $\bar{3}\cdot05$ with approximate 95 % fiducial limits at $\bar{3}\cdot32$ and $\bar{4}\cdot78$. The serum titres by the second, third and fourth estimates all fell within these fiducial limits and were $\bar{3}\cdot20$, $\bar{3}\cdot14$ and $\bar{3}\cdot23$.

https://doi.org/10.1017/S0022172400019586 Published online by Cambridge University Press

THE ANTIGENIC COMPARISON OF STRAINS OF INFLUENZA VIRUS

(i) Six strains of influenza virus were chosen which had previously been compared by a complement fixation technique (Fulton & Dumbell, 1949). The two A strains were WS and PR 8, the A′ strain was BAR (1947), the swine influenza strain was Shope 15, and the B strains were represented by Lee and CRA (1946). Each strain was matched in a neutralization test with convalescent ferret sera; the results are shown in Table 2. It is clear that the relationships disclosed by this

Table 2. *The antigenic comparison of six strains of influenza virus: titres of specific neutralizing antibodies*

Strains	Haemagglutinin titre*	Virus ID 50 (mm.³ of seed × 10^{-6})	Ferret sera: WS	PR 8	BAR	SHO	Lee	CRA	Normal
WS	$\bar{3}\cdot2$	50	**$\bar{4}\cdot4$**	$\bar{3}\cdot5$	$\bar{2}\cdot0$	$\bar{3}\cdot3$	0	0	0†
PR 8	$\bar{4}\cdot9$	316	$\bar{3}\cdot6$	**$\bar{5}\cdot5$**	0	0	0	0	0
BAR	$\bar{4}\cdot9$	10	0	0	**$\bar{3}\cdot05$**	0	0	0	0
SHO	$\bar{3}\cdot8$	15,000	0	0	0	**5·8**	0	0	0
Lee	$\bar{4}\cdot9$	4,000	0	0	0	0	**$\bar{4}\cdot7$**	$\bar{3}\cdot1$	0
CRA	$\bar{3}\cdot2$	4,000	0	0	0	0	$\bar{3}\cdot4$	**$\bar{4}\cdot7$**	0

* Salk pattern test: chick erythrocytes 0·25 % final.
† A serum titre of 0 means 'less than $\bar{2}\cdot0$'.

Table 3. *Heterologous relationships of the six strains of influenza virus at 10^{-2} serum levels*

Negative cups out of ten.*

Strains	Ferret sera: WS	PR 8	BAR	SHO	Lee	CRA	Normal
WS		9	5	8	4	4	1
PR 8	10		0	2	1	1	0
BAR	0	1		0	0	1	0
SHO	4	3	2		0	0	0
Lee	0	0	0	0		10	0
CRA	0	0	0	0	8		0

* A negative cup means that the virus has been neutralized in that cup

neutralization test are very similar to the relationships found by complement fixation, though the neutralization test is, as was to be expected, more strain specific. It is significant that the BAR strain which, under these conditions, is the most highly infective of the six strains is also the most difficult to neutralize.

Finer relationships are detected by considering the sets of ten replicates of the immune sera diluted 10^{-2} and combined with the various heterologous virus strains. The negative cups in these sets are recorded in Table 3. The presence of one negative cup in the set of ten is of no significance, as the virus level has been chosen so that the non-specific inhibitory power of the sera may occasionally cause this. But the four negative cups in each of the sets with Lee and CRA serum and WS virus suggest a specific serological relationship between the WS virus strain

https://doi.org/10.1017/S0022172400019586 Published online by Cambridge University Press

and the two B strains. The evidence presented here is only suggestive, but it confirms the slight unilateral relationship already described in the comparison by complement fixation. The belief that the A and B strains are quite distinct antigenically seems to have been based mainly on comparisons of PR 8 and Lee.

To make certain that this strain of WS had not at some time become contaminated with a strain of B virus, Dr Isaacs purified it by making two successive limit dilutions in eggs. The new strain of WS, which may be referred to as WS-2, was then examined by the neutralization technique. A virus pool prepared from it had an ID 50 of 40×10^{-6} mm.^{3}. Sets of ten replicates with 10^{-2} dilution of the same immune ferret sera gave no negative cups with normal serum, no negative cups with Lee serum, but seven negative cups with CRA serum. This result suggests that if there is an antigenic relationship between the WS strain and some of the Influenza B viruses, it is not a simple one. It is possible to speculate that the different elements composing the WS virus clone have different degrees of relationship to some Influenza B viruses.

Table 4. *The antigenic comparison of two strains of Influenza A′ virus*

	Titres of specific neutralizing antibodies			
			Ferret sera	
Strains	Haemagglutinin titre*	Virus ID 50 (mm.^{3} of seed $\times 10^{-6}$)	BAR	RHO
BAR	$\bar{4}{\cdot}9$	10	$\bar{3}{\cdot}05$	0†
RHO	$\bar{3}{\cdot}5$	4000	$\bar{4}{\cdot}87$	$\bar{5}{\cdot}8$

* Salk pattern test: chick erythrocytes 0·25 % final.
† A serum titre of 0 means 'less than $\bar{2}{\cdot}0$'.

(ii) Two strains of Influenza A′ virus, BAR and RHO, which have already been found to be identical by the complement-fixation test, were compared by the neutralization technique. The results are shown in Table 4. By this technique the two strains are not identical. The greater specificity of the neutralization test may well detect differences which the complement fixation disregards, but it is unlikely that this is the whole explanation. Although the higher value of the Bar serum titre with the RHO virus is just within the fiducial limits of the homologous estimate, it is suggested that the neutralization test measures not only the antigenic structure of the virus but also its infectivity under the defined conditions. For this reason especially, the complement-fixation technique is preferred for the antigenic comparison of strains.

DISCUSSION

The simplest explanation of the mechanism of the virus-neutralization test described here is to suppose that, during the preliminary incubation of the virus-serum mixture, a certain proportion of the virus is neutralized by the antiserum; when the piece of chorio-allantoic membrane is added, infection will occur if the amount of free virus is more than one ID 50. But if this were the whole truth it would be difficult to understand why the sensitivity of the test should be

enhanced by prolonging the time the membrane is left in the virus-serum mixture. It has proved easy to show that there is some specific neutralization even if the pieces of membrane are pre-infected before being transferred to the immune serum dilution for 18 hr. Therefore it is certain that the immune serum is also neutralizing the virus after adsorption of the virus on to the membrane has occurred. This secondary neutralization need not imply actual combination with virus already specifically adsorbed to or inside cells, but may simply reflect the binding of new virus from cells first infected which would otherwise have been free to infect other cells.

It is probable that the neutralization titre of a serum against its homologous virus is determined not only by the absolute amount of specific antibody present, but also, to a minor extent, by the infectivity of the strain used in the test; for the neutralization technique measures an elusive quality of 'infectivity' as well as antigenic structure. This complication is analogous to the differences in virus avidity noticed in the haemagglutinin-inhibition test (Hirst, 1943).

It is believed that the neutralization test and the complement-fixation test measure the same antibody population, but different fractions of it which have a partial overlap. The antibody spectrum is conceived as due both to variation in the goodness of fit of the antibody molecules with the native antigen, and to the breakdown of the native antigen in the course of the natural defensive processes of the convalescent animal, giving rise to a whole range of antibodies for the antigenic degradation products (Fulton & Begg, 1946). In the neutralization test the only fraction of antibodies which can be measured is that which has a good fit with the native antigen. The complement-fixation test will, in addition, measure other antibodies, the degree of overlap with the neutralization test depending on the stability of the antigen and on the way it has been purified and preserved.

If this concept is adopted, the neutralization test is seen to be not as well suited to the antigenic comparison of virus strains as the complement-fixation technique; the neutralization test is too specific and, in consequence, important relationships between strains will pass unobserved. However, because of its specificity, the neutralization technique may be useful for proving the virtual identity of two virus strains; it may also be useful in the selection of strains for a vaccine, provided it can be shown that the virus-neutralizing antibodies measured in the laboratory test are the same as the protective antibodies.

SUMMARY

A method of titrating influenza virus-neutralizing antibodies is described. This method is based on the technique for cultivating virus in fragments of chick chorio-allantoic membrane using a special plastic tray with cups for 100 replicates. The neutralization test cannot be made directly by adding the antiserum to the cups in which the virus is growing in the membrane fragments because there is a significant non-specific inhibitory effect. Therefore, use is made of the observation that virus added to the cups is rapidly fixed by the membrane so that, after a short time, the membrane may be washed and transferred to a fresh cup which will later be found to contain the new virus released from the membrane.

In the neutralization test the membrane fragments are exposed to virus-serum mixtures for 18 hr. and then transferred to other cups without serum to determine which pieces have become infected. The titre of neutralizing antibodies is defined as the serum dilution which will protect from infection with 100 ID 50 of virus, half of a large number of replicate pieces of membrane.

The neutralization technique has been applied to the antigenic comparison of six strains of influenza virus. The results support the relationships disclosed between the six strains by a complement-fixation technique. It is suggested, however, that the neutralization technique, because of its greater specificity, and because of its dependence on the infectivity of the strain, is not as satisfactory as the complement-fixation test for strain comparisons. Apart from its use for titrating neutralizing antibodies, the technique may also be of value for proving the virtual identity of two virus strains and in the selection of strains for a vaccine.

It is a pleasure to acknowledge the help given by Dr Isaacs of the WHO Influenza Centre. Not only did he provide many of the virus strains and antisera, but he also undertook the purification of the WS strain.

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(*MS. received for publication* 26. XII. 51.)