A COMPARISON OF THE SOLUBLE ANTIGEN PRODUCTION BY TISSUES INFECTED WITH PREPARATIONS OF EXTRACELLULAR AND INTRACELLULAR INFLUENZA VIRUS

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(With 2 Figures in the Text)

Many workers have shown that the intracellular form of influenza virus differs from the extracellular infective form (Hoyle, 1948, 1950; Henle & Henle, 1949; Burnet & Lind, 1951; Wyckoff, 1953). Extracellular virus has high infectivity in relation to its haemagglutinin and complement-fixing antigen titres. Intracellular virus, obtained by making tissue extracts during the latent period after infection, is very deficient in infectivity when compared with extracellular virus of similar haemagglutinin content.

Infectivity is usually titrated in eggs by the following technique. Eleven-day-old fertile hens' eggs are inoculated intra-allantoically with a range of dilutions to determine the amount required to infect half of them. Infection is recognized by the appearance of haemagglutinin in the allantoic fluid after 72 hr. incubation. Alternative ways of measuring infectivity can be devised. For example, complement-fixing soluble antigen is consistently produced in the chorioallantoic membranes of eggs infected with influenza virus. When large doses of virus are injected soluble antigen is readily detected at 6 hr. This observation can be used as the basis of a technique for measuring infectivity. Hoyle (1950) has already briefly reported the use of the method, and more recently Fulton & Isaacs (1953) have shown that soluble antigen production after chorionic inoculation can be used to measure virus infectivity.

When the two methods of measuring infectivity are compared using fully infective extracellular virus, they give essentially similar results. Intracellular virus gives very different results with the two methods. These differences are described and discussed.

MATERIALS AND METHODS

The D.S.P. strain of influenza A, first isolated in 1943 and maintained since then in eggs, was used. Eggs were inoculated with a small dose of virus, and 20–40 hr. later the allantoic fluid was harvested. This fluid was used as extracellular virus.

Intracellular virus was prepared by inoculating batches of eggs with a large dose of virus (0·4 ml. of infected allantoic fluid) and harvesting the membranes after 4½ hr. incubation. The embryos were tipped out and the membranes were washed with saline, twice in the shell and once in a Petri dish, and then cut into five or six pieces and placed in 0·5 ml. of saline containing 500 units of penicillin per ml. and frozen and thawed 3 times. The final thawing at 37° C. was prolonged for 6 hr. to

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allow elution of all the haemagglutinin (Hoyle, 1952). The membranes were finally removed by centrifugation.

Infectivity titrations were done by making serial tenfold dilutions and inoculating 0·5 ml. into each of five eggs per dilution. After 72 hr. incubation the allantoic fluids were tested for haemagglutinin and the 50% end-point calculated by Thompson's (1947) method.

The amount of soluble antigen produced in the membrane at 6 hr. was determined by inoculating 1 ml. quantities of various dilutions of the virus preparations intra-allantoically into batches of four eggs. After 6 hr. the membranes were

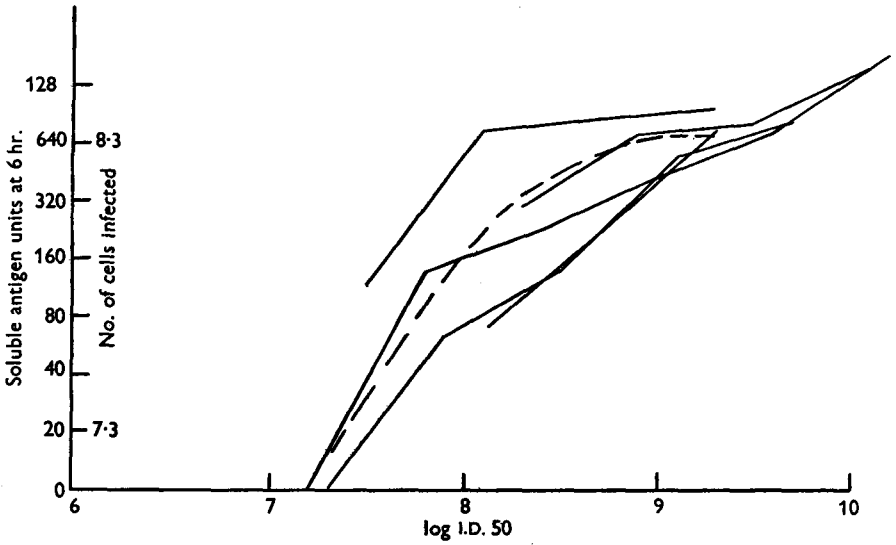

Fig. 1. The relationship between infectivity and soluble antigen production at 6 hr. for extracellular virus. The broken line represents the theoretical relationship between the size of the inoculum and the number of cells infected.

harvested as in the preparation of intracellular virus. Each membrane was harvested separately into 1 ml. of azide saline (10%M/8 sodium azide in normal saline). The membranes were frozen and thawed 3 times and then removed. The extracts were tested for soluble antigen by the complement-fixation technique described by Hoyle (1945), using human convalescent serum from the 1951 influenza epidemic. The results were recorded in soluble antigen units per membrane.

Haemagglutinin tests were done by a modification of Salk's (1944) method, using 1:250 guinea-pig red cells and reading the test after 2 hr. at room temperature.

RESULTS

Extracellular virus

When the infectivity of extracellular virus was compared with its ability to provoke soluble antigen formation, the relationship shown in Fig. 1 was found. This shows the average yield of soluble antigen per membrane plotted against the infectivity of inocula in terms of I.D. 50. In order to double the yield of soluble antigen about a fivefold increase in the dose of virus inoculated was required.

If large doses of virus were used, sufficient to infect all the cells, there was an average yield of 640 units of soluble antigen per membrane at 6 hr. Smaller doses infected fewer cells and proportionately less soluble antigen was produced. The relationship between the dose of virus inoculated and the number of cells infected is given by the formula $n = N\ (1 - e^{-x/N})$, where n is the number of cells infected, N is the total number of cells and x the dose of virus. The theoretical curve has been

Table 1. *A comparison of the haemagglutinin titre, infectivity and soluble antigen production at 6 hr. of extracellular and intracellular virus*

Extracellular virus			Intracellular virus		
Haemagglutinin titre	I.D. 50 per ml. × 10^6	Soluble antigen units at 6 hr. per membrane	Haemagglutinin titre	I.D. 50 per ml × 10^6	Soluble antigen units at 6 hr. per membrane
1280	6400	812	1280	20	382
320	1600	550	320	5	136
160	400	135	160	1·25	0
40	100	63	—	—	—
10	25	—	—	—	—

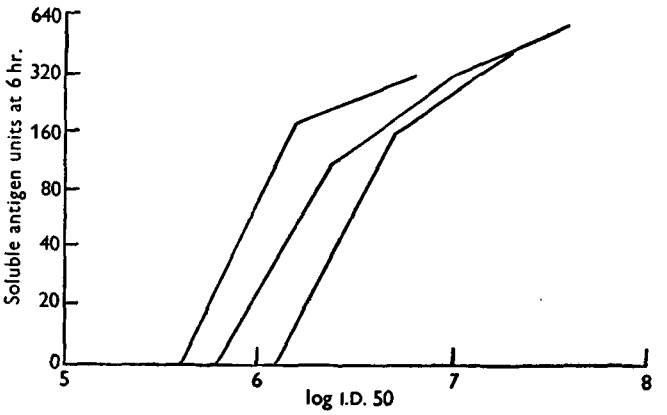

Fig. 2. The relationship between infectivity and soluble antigen production at 6 hr. for intracellular virus.

plotted on Fig. 1, assuming there are 200 million cells lining the allantoic sac. There is sufficiently close relationship between this theoretical curve and those relating soluble antigen production to infectivity of inoculum, to justify the conclusion that the amount of soluble antigen produced is related to the number of cells infected.

Intracellular virus

Intracellular virus of similar haemagglutinin titre to the extracellular form was prepared. The infectivity was so low that, from the results shown in Fig. 1, this virus would be expected to produce little or no soluble antigen in the chorio-allantoic membrane at 6 hr. However, as shown in Fig. 2 and in Table 1, considerable amounts of soluble antigen were produced by these preparations. Intracellular

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virus produces as much soluble antigen as extracellular virus, which is nearly 100 times as infective by the standard test, although less than that produced by extracellular virus of similar haemagglutinin titre. The curves in Fig. 2 are of rather different shape from those in Fig. 1, in that the amount of soluble antigen produced falls much more suddenly on dilution of the inoculum when this is intracellular virus.

DISCUSSION

The $4\frac{1}{2}$ hr. membrane extracts used in these experiments contained appreciable quantities of superficially adsorbed extracellular virus, in addition to the multiplying intracellular virus. Both components must play a part in the observed results, but their combined infectivity in the standard test is insufficient to explain the amount of soluble antigen produced at 6 hr. The extracts were made at $4\frac{1}{2}$ hr. so as to obtain preparations containing multiplying intracellular virus, but before any great rise in infectivity occurred. The results show that these extracts contain particles, presumably intracellular virus, which are capable of initiating infection as judged by testing for soluble antigen production at 6 hr. Assuming that for fully infective extracellular virus one particle per cell can initiate infection, an egg which receives one particle in an infectivity titration will become positive. If several particles per cell are required, a preparation containing the same number of particles will have a far lower infectivity, because many more particles will have to be introduced to ensure multiple infection of one of the 200 million cells lining the allantoic cavity. On the other hand, if measurable soluble antigen in the membrane is to be produced at 6 hr., large doses of virus have to be inoculated and multiple infection of cells probably occurs. Thus the most likely explanation of the experimental results is that the infective dose of intracellular virus is more than one particle per cell. This would also explain the sharp fall in soluble antigen production on dilution of the inoculum to a level at which presumably little multiple infection can occur.

This explanation is in accord with the ideas of Burnet (1953) and Hoyle (1953), that on entry into the cell influenza virus is broken down into smaller units which multiply independently and are later re-aggregated and excreted as fully infective virus. No decision can be made between the views of Burnet and Hoyle. Multiple infection may be required to ensure the presence of every genetic unit, or, as Hoyle suggests, the amount of soluble antigen introduced into the cell may be the critical factor.

The phenomenon is very similar to multiplicity reactivation as described by Henle & Liu (1951) for influenza virus partially inactivated by heat or ultraviolet light. They found that inactivated virus when inoculated in large doses produced more haemagglutinin and infective virus than would be expected from its infectivity in the standard test. Isaacs & Fulton (1953) have also been able to detect multiplicity reactivation by measuring the soluble antigen production following large doses of ultraviolet inactivated virus inoculated on to the chorion.

SUMMARY

1. The production of soluble antigen in the chorioallantoic membranes at 6 hr. can be used to measure the infectivity of influenza virus preparations.

2. Intracellular virus is much more infective when tested by soluble antigen production at 6 hr. than when tested by conventional methods.

3. It is suggested that intracellular virus differs from extracellular because more virus particles per cell are required to initiate infection.

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