Studies on the Mechanism of Neutralization of Influenza Virus by Antibody: Evidence that Neutralizing Antibody (Anti-haemagglutinin) Inactivates Influenza Virus in vivo by Inhibiting Virion Transcriptase Activity

By R. D. POSSEE, G. C. SCHILD and N. J. DIMMOCK

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K. and National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, U.K.

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SUMMARY

Influenza viruses, which had lost up to 99.999% infectivity by incubation with antibody (α) specific for the haemagglutinin (HA) or with monoclonal α-HA, attached to and penetrated chick embryo fibroblast (CEF) cells to the same extent as non-neutralized virus. Neutralized virus was also uncoated efficiently as shown by the accumulation of virion RNA in the nucleus and virion envelope in the cytoplasm. Polyacrylamide gel electrophoresis of virion RNA segments recovered from the nucleus or cytoplasm of cells inoculated with neutralized or non-neutralized virus showed that antibody did not potentiate degradation of RNA. However, these RNAs were not expressed since virus-induced proteins were not detected in cells to which neutralized virus had been added. Assay of virion transcriptase of neutralized virus in vitro showed that its activity was reduced up to sevenfold compared with non-neutralized virus, and annealing studies showed that no detectable transcription took place in vivo with neutralized virus. These studies support the conclusion that antibody directed specifically against the HA protein on the outer surface of the influenza virus particle neutralizes infectivity by inactivating virion transcriptase activity and it is suggested that antibody to HA brings about allosteric rearrangements in the HA molecule which are transmitted across the virus envelope to the interior of the particle.

INTRODUCTION

The interaction of virus with neutralizing antibody results in a loss of infectivity which can be measured by titration in animals or cells in culture. It has been shown for several groups of animal viruses that immunoglobulins (Ig) G, A and M all have neutralizing activity (Ogra et al., 1975) but IgG is probably the most important as it is present in the greatest amount and is distributed through the bloodstream, lymphatics and peripheral body fluids. Despite its obvious importance to immunity there is little firm evidence for any virus to indicate how neutralizing antibody renders a virus particle non-infectious. It is commonly held that antibody sterically hinders the initial association of neutralized virus with cellular receptors. This may well be true when a particle is saturated with antibody but such high concentrations of antibody are rarely physiological and it has been argued from the first-order reaction kinetics of inactivation that one antibody molecule on the surface of each particle is sufficient to neutralize its infectivity (Dulbecco et al., 1956; Mandel, 1960). There are also arguments that neutralization is a multi-hit process (for review, see Daniels, 1975; Della-Porta.
& Westaway, 1977) and it may be that with different viruses there is no unifying mechanism of neutralization.

The binding of antibody molecules to the surface of a virus particle does not necessarily reduce infectivity of influenza virus (Webster & Laver, 1967), adenovirus (Kjellén & Pereira, 1968) or T-even virus (Lanni & Lanni, 1953) and may even enhance it (Hawkes, 1964; Hawkes & Lafferty, 1967; Barrett, 1971; Halstead & O’Rourke, 1977; Kliks & Halstead, 1980; Peiris et al., 1981). An entirely new concept of neutralization was suggested by Mandel (1976) who showed that poliovirus existed in two reversible isoelectric conformations, only one of which was infectious, and that neutralizing antibody caused the virus to remain in the non-infectious state. This was interpreted as arising from allosteric changes in the protein subunits of the virus. A priori it would seem difficult to extrapolate this concept to the neutralization of enveloped viruses. However, it is possible that close structural connections between envelope proteins and those within the particle, such as with Semliki Forest virus (Garoff & Simons, 1974) and vesicular stomatitis virus (Dubrovi & Wagner, 1977; Mudd & Swanson, 1978) could be disturbed by antibody reacting at the virus surface.

The influenza viruses are particularly suited for investigating neutralization as there is considerable knowledge of the structure, antigenicity and functions of the components of the virion (see British Medical Bulletin, 1979; Tyrrell & Pereira, 1980; Laver & Air, 1980). There are two external envelope proteins, the haemagglutinin (HA), against which neutralizing antibody is directed, and the neuraminidase (NA). Neuraminidase antibody inhibits enzymic activity but does not neutralize (Webster & Laver, 1967) and thus provides a useful control for the effects of antibody attached to the virus particle. Recently, the use of monoclonal antibodies has advanced understanding of the antigenic structure of the HA (Gerhard, 1978; Gerhard & Webster, 1978; Gerhard et al., 1980) and 3Å resolution X ray diffraction studies of the HA and its binding to antibody have been published (Wiley et al., 1981; Wilson et al., 1981). Much is also known about molecular aspects of multiplication (see British Medical Bulletin, 1979; Tyrrell & Pereira, 1980) and of the initial events of attachment, penetration and uncoating (Huang, 1974; Stephenson & Dimmock, 1975; Hudson et al., 1978; Stephenson et al., 1978; Rott, 1980). In particular, the entry into the nucleus of the RNA, but not the envelope, of infecting virus particles provides a valuable means of discriminating between penetration into a cell and uncoating which may well be relevant to neutralization. It is the continuous evolution of the HA and NA which makes influenza viruses an ever present source of epidemic infection. An understanding of the mechanism of neutralization of influenza A viruses may lead to rational developments and improvements in the immunoprophylaxis of the disease.

**METHODS**

**Viruses and cells.** The avian strain of influenza virus A/Fowl plague virus/Rostock/34 (H7N1) (FPV/R) and a human strain X49 (H3N2), which is a reassortant cloned from the progeny of a mixed infection of A/England/864/75 (H3N2) and A/PR/8/34 (H1N1), were used. Viruses were grown by inoculation of about 10⁵ p.f.u. into the allantoic cavity of 11-day-old embryonated hens’ eggs and incubated for 22 h at 37 °C (FPV/R) or 48 h at 33 °C (X49). Primary chick embryo fibroblast (CEF) cells, prepared according to Morser et al. (1973), were used for the majority of experiments and to plaque assay FPV/R under 0-9% agar in medium 199 containing 2% newborn calf serum. X49 was assayed on monolayers of MDCK (canine kidney) cells under an agar/199 medium overlay containing no serum and 2-5 μg/ml trypsin–TPCK (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) which cleaves virus HA and renders the progeny virus infectious (Appleyard & Maber, 1974; Lazarowitz & Choppin, 1975; Klenk et al., 1975).

**Uptake of virus into CEF cells.** Under optimum conditions, up to 80% of the cell-
associated virus RNA enters the nucleus by 1 h after infection at 4 °C but both CEF cells and virus preparations are variable in this regard. Consequently, freshly prepared CEF cells were stored under liquid nitrogen while monolayers were screened for their ability to take up virus (Stephenson et al., 1978); it was also necessary to use virus preparations of low particle : infectivity ratio to achieve high levels of nuclear association of virion RNA (see Fig. 1).

**Preparation of nuclear and cytoplasmic fractions.** All cellular fractionations were performed in duplicate using Dounce homogenization or a modification of the nuclear monolayer (NML) method (Tsai & Green, 1973; Hudson & Dimmock, 1977) to reduce the possibility of methodological artefacts. There was always good agreement between the two methods.

The homogenization procedure was essentially that of Crampton & Woodland (1979): cells were scraped from culture dishes into phosphate-buffered saline (PBS) and pelleted before resuspending in 1.25 ml ice-cold homogenization medium (0.3 μM sucrose, 2 mM-magnesium acetate, 3 mM-CaCl₂, 10 mM-tris–HCl pH 8, 0.1% Triton X-100, 0.5 mM-dithiothreitol) at about 3.6 × 10⁷ cells/ml. After incubation on ice for 5 min the cells were subjected to 20 strokes in a tight-fitting stainless-steel Dounce homogenizer. An equal volume of ice-cold centrifugation medium (2 M sucrose, 5 mM-magnesium acetate, 10 mM-tris-HCl pH 8, 0.5 mM-dithiothreitol) was added and a further 5 strokes given. This mixture was layered over 8 ml centrifugation medium and centrifuged at 4000 g for 1 h in a Sorvall RC-5B centrifuge. The supernatant was removed and the nuclei resuspended in 25% glycerol, 5 mM-magnesium acetate, 50 mM-tris–HCl pH 8, 5 mM-DTT and 0.1 mM-EDTA.

For NML fractionation cytoplasm was extracted from cell monolayers at 4 °C using two washes of 5 min each with 2% (w/v) Nonidet P40 (NP40; BDH) in 0.25 M-sucrose, 10 mM-tris–HCl pH 7.4, 1 mM-MgCl₂, leaving nuclei attached to the Petri dish. These were scraped into the same buffer, without NP40, rinsed vigorously on a vortex mixer and pelleted at 160 g for 5 min at 4 °C. The supernatant was pooled with the NP40 fraction. CEF cells fractionated easily and give a high yield of clean nuclei by either method. The NML procedure has a number of advantages in that its speed and simplicity help to minimize the possibility of damage or subsequent leakage from the nuclei and allow several monolayers to be fractionated simultaneously.

**Evaluation of cellular fractionation.** Since the preparation of clean nuclei depends very much on the experimenter we have re-examined some parameters of nuclear purity for the series of experiments described in this report. The following findings are in agreement with our previous experience of cell fractionation techniques (Stephenson & Dimmock, 1974, 1975; Hudson & Dimmock, 1977; Flawith & Dimmock, 1979). Little newly synthesized HA was associated with the nuclei (3.3%); stably labelled DNA and pulse-labelled RNA were nuclear (94 and 93% respectively) while stably labelled RNA was cytoplasmic (90%). Whereas up to 80% virion RNA of our infecting influenza virus (FPV/R) accumulated in the nuclei of cells inoculated at 4 °C, 92% lipid envelope ([3H]choline) remained in the cytoplasm (Stephenson & Dimmock, 1975; Hudson et al., 1978; Mark et al., 1979; Tables 1 and 2). The RNA of Semliki Forest virus (SFV), which can infect CEF cells at 4 °C (Stephenson et al., 1978), was predominantly cytoplasmic (92%). Clean nuclei are much easier to prepare from CEF cells than from other cell types and phase-contrast microscopy showed no contamination with cytoplasmic tags. The results shown below were obtained by the nuclear monolayer method of fractionation but identical results were achieved in all experiments using Dounce homogenization (data not shown).

**Radiolabelling and purification of virus.** [32P]phosphate (32P)-labelled influenza virus was prepared in roller bottles containing 2 × 10⁸ CEF cells (Crumpton et al., 1978). Monolayers were incubated in phosphate-free Glasgow minimal essential medium (GMEM) containing 5% dialysed calf serum for 24 h before infecting with virus at a multiplicity of 0.1 p.f.u./cell.
After 1 h the inoculum was removed and replaced with 70 ml medium containing 0.5% calf serum and 10 mCi $^{32}$PO$_4^{3-}$ (Amersham International); incubation was continued for 20 h. The virus envelope was labelled with 0.5 mCi [methyl-$^3$H]choline chloride (Amersham International; sp. act. 77 Ci/mmoll in the same way but using complete GMEM. Virus was precipitated from tissue culture fluid on ice with 60% saturated (NH$_4$)$_2$SO$_4$ and purified by sucrose velocity-gradient centrifugation (Dimmock et al., 1977) except that no unlabelled carrier virus was added. One monolayer yielded about $10^4$ haemagglutinating units (HAU) having on average $4 \times 10^7$ ct/min $^{32}$P. Unlabelled virus from allantoic fluid for use in transcriptase assays was pelleted at 30000 g for 1 h before purification on velocity and equilibrium gradients (Kelly & Dimmock, 1974). SFV was grown and radiolabelled with [$^3$H]uridine (sp. act. 26 Ci/mmol) in roller bottles of CEF cells. Virus was subsequently purified as described by Bruton & Kennedy (1976).

Antisera. Antiserum to the HA (H7) of FPV/R was raised in rabbits inoculated with A/FPV/Dutch/27 (H7N7) (FPV/D) which has an HA antigen closely related to that of FPV/R and an antigenically unrelated NA. $10^5$ HAU of purified FPV/D were injected intravenously at days 0 and 21 and serum obtained by bleeding at day 27. Antibodies to typespecific antigens nucleoprotein (NP) and matrix (M) protein were removed by adsorption with ether-disrupted A/HK/1/68 (H3N2) after which no anti-NP or M antibody could be detected by immuno-double-diffusion (Dimmock, 1969). Rabbit antibody to X-55 (H1N1), a reassortant cloned from the progeny of a mixed infection of A/Swine/1976/31 (H1N1) and A/PR/8/34 (H1N1), was used to inhibit the neuraminidase of FPV/R. This serum was not adsorbed.

A hybridoma cell line (171/7) secreting monoclonal IgG which neutralized X49 virus, kindly provided by Dr R. G. Webster, was made by fusing together spleen cells from Balb/c mice immunized with A/Texas/1/77 (H3N2) and MOPC-21 cells (Kohler & Milstein, 1975; Koprowski et al., 1977). The HA of A/Texas/1/77 is antigenically closely related to the H3N2 parent of X49. Ascitic fluid containing antibody was obtained by intraperitoneal inoculation of hybridoma cells. Rabbit and mouse immunoglobulins were purified by affinity chromatography on a column of protein A–Sepharose (Pharmacia) and had haemagglutination-inhibiting titres of 1:4500 for $\alpha$-H7HA (against FPV/R) and 1:9000 for $\alpha$-H3HA (against X49 virus).

Extraction and analysis of RNA by polyacrylamide gel electrophoresis (PAGE). These were performed according to Crumpton et al. (1978).

RNA hybridization. RNA was extracted from cells infected with radiolabelled virions and self-annealed according to the Stephenson & Dimmock (1975) adaptation of the method of Bean & Simpson (1973). The extent to which the input $^{32}$P-labelled RNA was protected from digestion with ribonucleases A and T$_1$ was indicative of the amount of transcription which had taken place.

$[^{35}S] $methionine radiolabelling of infected cells and analysis of polypeptides by PAGE. CEF monolayers were pulse-labelled for 30 min with 25 $\mu$Ci $[^{35}S]$methionine (sp. act. 800 $\mu$Ci/mmoll; Amersham International) in 0.5 ml Earle's saline pH 7.4. Cells were solubilized in 2% SDS, 5% 2-mercaptoethanol, 5% glycerol, 10 mM-tris–HCl pH 7.4 and analysed on 10 to 30% linear polyacrylamide gradient gels (Cook et al., 1979) using the buffer system of Laemmli (1970). Following electrophoresis, gels were dried and exposed for autoradiography using Fuji Rx X-ray film (Fuji Photo Film Co., Tokyo, Japan).

Transcriptase assay. The in vitro assay as previously described by Bishop et al. (1971), Skehel (1971), Penhoet et al. (1971), and Chow & Simpson (1971) was optimized for FPV/R as described by Minor & Dimmock (1979). The dinucleotide adenylyl(3'-5')guanosine (ApG) (Sigma) was used at a concentration of 0.4 mM in some assays to stimulate transcription (McGeoch & Kitron, 1975; Plotch & Krug, 1977).
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Figure 1. Association of 32P-labelled FPV/R RNA with the nucleus as a function of the p.f.u./HA ratio. These data were obtained with a single batch of CEF cells infected at 4 °C. Each point represents a different preparation of virus with a fortuitously different p.f.u./HA ratio. Conditions of infection and fractionation are described in Table 2 and the curve plotted by regression analysis.

Table 1. Nuclear association of 32P-labelled FPV/R RNA*

<table>
<thead>
<tr>
<th>Cell- associated</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>% cell-</td>
<td>Ct/min (%)</td>
<td>Ct/min (%)</td>
</tr>
<tr>
<td>+plg</td>
<td>10-5</td>
<td>67951 (71)</td>
<td>27675 (29)</td>
</tr>
<tr>
<td>+α-H7HA</td>
<td>12-5</td>
<td>92970 (69)</td>
<td>42427 (31)</td>
</tr>
</tbody>
</table>

*4.2 x 10^7 p.f.u. FPV/R (756 HAU) in 325 μl were incubated for 1 h at 25 °C with an equal volume of IgG (1.1 mg) purified from rabbit α-H7HA serum adsorbed to remove type-specific antibodies (see Methods) or with the equivalent amount of IgG obtained from a preimmunization serum (plg). The virus–antibody mixture was cooled to 4 °C and 300 μl inoculated on to duplicate cold CEF monolayers (9 x 10^6 cells/dish). After 1 h cells were rinsed thoroughly with cold PBS, fractionated by NML assay and TCA-precipitable radioactivity was determined. Dilutions of the same virus–antibody preparation were assayed for infectivity to determine the extent of neutralization.

† TCA-precipitable ct/min.

RESULTS

Association of the RNA of neutralized virus with the nucleus

The majority of the RNA of cell-associated FPV/R enters and remains in the nuclei of CEF cells held at 4 °C (Stephenson & Dimmock, 1975) and this provides a very useful quantitative measure for entry and uncoating of neutralized virus. However, nuclear association of the RNA of non-neutralized virus was a function of its specific infectivity (Fig. 1). Hence, it is important to use preparations of the highest infectivity:particle ratio. Fig. 1 also suggests that the observed accumulation of virion RNA in the nucleus is related to the infectious process. However, when FPV/R was neutralized to 99.999% at 25 °C with α-H7HA and then cooled and inoculated on to CEF monolayers at 4 °C, neutralized virus attached to cells to the same extent as non-neutralized virus and their RNAs became distributed between nucleus and cytoplasm in an identical manner (Table 1). Table 2 shows that the virus envelope of non-neutralized and neutralized FPV/R remained cytoplasmic. Clearly, virus which was extensively neutralized interacted with cells in the same way as infective virus.

Although virion RNA from cells inoculated with neutralized virus was trichloroacetic acid (TCA)-precipitable it was possible that neutralization has potentiated a selective degradation process. This was tested by extracting RNAs from nuclear and cytoplasmic fractions and
Fig. 2. PAGE of $^{32}$P-labelled virion (FPV/R) RNA extracted from nuclear (N) and cytoplasmic (C) fractions of CEF cells inoculated at 4 °C with radiolabelled non-neutralized ($p.f.u.: HA = 10^5$°) or neutralized FPV/R incubated with preimmunization (plg) or $\alpha$-H7HA IgG respectively. Conditions of neutralization and infection are described in Table 1. V = marker RNA extracted from FPV/R virions.

Fig. 3. PAGE of CEF cells inoculated with neutralized FPV/R (+ $\alpha$-H7HA) or with non-neutralized virus ($p.f.u.: HA = 10^5$°) incubated with preimmune serum (plg) or with PBS (S). Conditions of neutralization are the same as in Table 1. Cells were initially incubated with virus for 1 h at 4 °C, then the inoculum was removed and warm medium added. After 5 h, cells were pulsed with 25 $\mu$Ci $[^{35}S]$-methionine in 0.5 ml medium for 30 min and harvested. Nomenclature of virus proteins follows standard conventions (Inglis et al., 1976; Kilbourne et al., 1972; Lamb & Choppin, 1976; Palese et al., 1977). NI = non-infected cells; V = $[^{35}S]$methionine-labelled FPV/R virions (Dimmock et al., 1977); a = actin.

Table 2. Cytoplasmic uncoating of $^3H$ choline-labelled FPV/R*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Cell-associated</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ct/min</td>
<td>Ct/min</td>
<td>p.f.u./ml</td>
</tr>
<tr>
<td>+plg</td>
<td>31</td>
<td>325 (10)</td>
<td>2891 (90)</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>+$\alpha$-H7HA</td>
<td>33</td>
<td>304 (9)</td>
<td>2917 (91)</td>
<td>$9 \times 10^2$</td>
</tr>
</tbody>
</table>

* Experimental conditions are described in Table 1.
Fig. 4. Effect of neutralizing and non-neutralizing antibody on virion transcriptase activity in vitro. (a) Concomitant neutralization and inhibition of FPV/R transcriptase. 1700 HAU were incubated prior to assaying for transcription with buffer solution (○) or with 670 µg immunoglobulin (α-H7HA) purified from antiserum adsorbed (●) or not (Δ) to remove type-specific antibodies as described in Methods. Infectivity was neutralized 99.999% under conditions described in Table 1. (b) Failure of anti-neuraminidase immunoglobulin (α-NA) to inhibit the in vitro transcriptase activity of FPV/R. 5623 HAU were incubated with PBS (○), α-N1NA (▲) and α-H7HA (●). Infectivity was neutralized by α-H7HA by 99.8% but not by α-NA (365 µg). (c) Concomitant neutralization and inhibition of transcriptase activity of X49 virus by monoclonal α-H3HA. 1259 HAU were incubated with monoclonal α-H3HA (●) immunoglobulin or the equivalent amount of non-immune immunoglobulin (100 µg; ○). Infectivity was neutralized by 99.999%. (d) Failure of ultrasonication to increase the in vitro transcriptase activity of neutralized FPV/R. 3374 HAU were incubated with buffer solution or purified monospecific α-H7HA IgG (1.34 mg). A sample was removed from each to estimate neutralization by plaque assay and the remainder divided equally, made 0.55% with respect to NP40, and either sonicated or left untreated before assaying for transcription. ■, Buffer; □, buffer + sonication; ●, α-H7HA; ○, α-H7HA + sonication.
analysing them by PAGE. Fig. 2 shows no evidence of degradation of virus RNA segments although we cannot rule out the loss of a few terminal nucleotides.

**Neutralized virus does not direct protein synthesis in vivo**

Loss of infectivity caused by neutralizing antibody could be the result of an inability to synthesize one or more virus proteins. However, when cells were inoculated with neutralized virus and pulse-labelled with [³⁵S]methionine no virus proteins were detected (Fig. 3). Therefore, neutralizing antibody appeared to induce some defect in multiplication at a stage between the accumulation of virion RNA in the nucleus and the de novo synthesis of virus proteins. Accordingly, we investigated virion transcriptase function.

**Transcriptase activity in vitro of neutralized virus**

Fig. 4 (a) shows that the in vitro transcriptase activity of neutralized FPV/R was reduced by sevenfold compared with virus incubated under identical conditions with PBS. This was a specific effect as the transcriptase activity of virus incubated with the same amount of α-NA immunoglobulin was not significantly inhibited (Fig. 4 b).

Three lines of evidence suggest that the reduction in RNA synthesis was not the result of contaminating ribonuclease: (i) purified virion RNA remained undegraded when mixed with neutralizing antibody (data not shown), (ii) incubation with α-NA (Fig. 4 b) or a control immunoglobulin preparation (Fig. 4 c) failed to inhibit transcriptase activity and (iii) transcriptase activity of neutralized virus was still inhibited after the virus had been separated from free antibody and putative ribonuclease by centrifugation through a sucrose spacer (data not shown). A possible explanation of the inhibition of transcription by neutralized virus was that the α-H7HA preparation contained antibodies to the transcriptase which had not been removed by the adsorption process. To answer this criticism, X49 virus was incubated with monoclonal α-H3HA, which reduced infectivity 99.999%, or with an equivalent amount of non-neutralizing immunoglobulin. Fig. 4 (c) shows that the transcriptase activity of neutralized X49 was reduced by ninefold. A more remote possibility was that the monoclonal antibody reacted with determinants of the transcriptase complex. This was checked by separating free antibody from X49 plus bound antibody by centrifuging the neutralization mixture over 5% sucrose and assaying the transcriptase activity of the pelleted virus. This was found to be reduced relative to the control suggesting that cross-reacting antibody was not responsible for inhibition of the enzyme (data not shown).

The possibility that virus was aggregated by antibody and that this resulted in the observed loss of transcriptase activity was unlikely as the concentration of virus was relatively low and in fact we found that ultrasonication did not affect the infectivity remaining after neutralization. Disruption of putative aggregates with ultrasound immediately before assaying for transcription did not increase activity of neutralized virus (Fig. 4 d). Furthermore, the RNA of neutralized FPV/R prepared for transcriptase assay in 0-25% NP40 was completely susceptible to RNase, suggesting that antibody did not prevent access to the small molecules required for transcription (data not shown).

**Transcriptase activity in vivo of neutralized virus**

The relevance of in vitro inhibition of transcriptase activity of neutralized virus to the in vivo situation was examined by inoculating CEF monolayers with [³²H]-labelled FPV/R incubated with increasing dilutions of neutralizing antibody. Transcriptase activity was measured by the development of RNase resistance by α²p-labelled RNA from inoculated virions, which increased with time up to 2 h post-infection as complementary RNA was synthesized (Fig. 5). The acquisition of RNase resistance of neutralized virus was measured at 3 h post-infection and is shown in Fig. 6 in relation to the extent of neutralization. With
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Fig. 5. Time course of transcription of FPV/R (p.f.u.: HA = 10^5) in CEF monolayers. Cells were infected with ^3P-labelled virus. RNA was extracted after incubation at 37 °C for the times indicated, self-annealed and digested with ribonucleases A and T1 to remove single-stranded RNA.

Fig. 6. Transcriptase activity in vivo of ^3P-labelled FPV/R (p.f.u.: HA = 10^4) incubated with dilutions of α-H7HA or with preimmunization antibody (pig) equivalent to undiluted α-HA, for 1 h at 25 °C. The percentage neutralization of virus infectivity is shown on the same axis (O). RNA was extracted after 3 h incubation at 37 °C, and treated as in Fig. 5. Ribonuclease resistance is expressed as a percentage of virus incubated with preimmunization antibody (●).

serum dilutions (1:1 to 1:10) which neutralized infectivity by 99.999% or greater there was no detectable transcriptase activity. A further threefold dilution of antiserum almost abolished neutralization and restored full transcriptase activity. Thus, there appeared to be a close correlation between neutralization of infectivity and inhibition of the transcriptase.

DISCUSSION

Influenza virus attaches to and enters human and CEF cells held at 4 °C by an energy-independent micropinocytosis (Hackeman et al., 1974; Patterson et al., 1979). Virus is uncoated at 4 °C since virion RNA and associated proteins enter the nucleus while the envelope remains in the cytoplasm (Stephenson & Dimmock, 1975; Hudson et al., 1978). Virion RNA similarly enters nuclei at 25 °C and 37 °C but at 4 °C it does move out of the nucleus and is not transcribed until the temperature is raised to a physiological level (Stephenson & Dimmock, 1975). This system has allowed us to determine the fate of neutralized virus and to show that neutralized virus attaches to and is taken up by cells to the same extent as non-neutralized virus although at a slightly slower rate (Possee & Dimmock, 1981). The same results were seen with different subtypes of influenza virus (avian and human) and with neutralizing antibody from different species. Rabbit anti-FPV/R HA was obtained by immunization with whole virus (FPV/D) and presumably contains antibodies against many of the 40 or so epitopes present on the HA (Gerhard, 1978) and anti-X49 HA antibody used is a mouse monoclonal IgG antibody specific for H3HA protein. There was no difference in the findings from any experiment which could be attributable to the type of antibody. Neither antibody inhibited or enhanced the uptake of neutralized virus into cells. The second surprising finding with neutralized virus was that its RNA associated with the nucleus to the same extent as that of non-neutralized virus and was
apparently undegraded. This we interpret as showing that neutralized virus is uncoated normally.

It is of interest to note that although different batches of $^{32}$P-labelled FPV/R attached to cells equally well, virion RNA did not become nucleus-associated with equal efficiency and we observed (Fig. 1) that high nuclear localization of virion RNA correlated with a high infectivity:particle ratio. Thus, the efficiency of uptake into the cell and/or transport from the cytoplasm to the nucleus appears to play some role in determining the infectivity of the virus. However, the RNA of neutralized influenza virus is transported to the nucleus as efficiently as that of non-neutralized virus, which suggests some role for antibody in neutralization of influenza viruses other than inhibition of attachment, penetration, uncoating and transport of virion RNA. Since neutralized virus synthesized no new virus proteins we concluded that neutralizing antibody inhibited some step in the pathway leading to synthesis of viral mRNA and studies in vitro indicated that it is the synthetic activity of the transcriptase which is non-functional. Of greater significance and in complete accord was the finding that in vivo neutralized virus failed to transcribe complementary or mRNA.

We concluded, therefore, that α-HA inhibits transcription of virus mRNA. The composition of the transcriptase is unknown since faithfully transcribing enzyme has not been purified free of template (Rochovansky, 1976) and the transcription complex comprises virion RNA, nucleoprotein (NP) and P proteins. Genetic studies also show that NP, P1, P2 and P3 all have a role in transcription (Barry & Mahy, 1979). One possible explanation for our data is that HA and the transcriptase complex have common antigenic determinants. However, transcriptase activity of neutralized virus in vitro was reduced to the same extent after it had been separated from free antibody by centrifugation although it cannot be completely ruled out that antibody eluted from HA and bound to the transcriptase during the assay. Nevertheless, it would strain coincidence to suggest that the one antigenic determinant to which the monoclonal antibody binds is present on both HA and transcriptase. Arguments about the effects of free antibody have less relevance in vivo as antibodies do not enter cells. What happens to antibody which enters the cell bound to virus is not known and we are investigating this aspect.

Thus, in our view, the most likely hypothesis is that transcriptase activity is inhibited as the result of antibody binding to the HA. While there are undoubtedly many steps involving both synthetic and non-synthetic (e.g. transport) events leading to production of mRNA, inhibition of transcription in vitro argues that α-HA affects the synthetic activity of the transcriptase. Krug et al. (1981) have defined three activities involved in the synthesis of virus mRNA in vivo: recognition of a methylated cap structure on a cellular mRNA, cleavage of a 5'-oligonucleotide and its extension as a complement to vRNA. The failure of neutralized virus to transcribe in vitro would suggest that the last of these activities was defective. Our hypothesis requires that the HA penetrates the virus envelope and is in physical contact with the transcriptase within the particle but as the data above (Tables 1 and 2) show that the virion envelope remains in the cytoplasm while the RNA enters the nucleus, it follows that inhibition of the transcriptase is not dependent upon the integrity of the neutralized virus particle. Sequence studies on the HA molecules of FPV/R, H2N2 and H3N2 strains suggest that alignment of the most hydrophobic sequence of amino acids in the carboxyl region of HA2 with the virus envelope would leave the 11 terminal residues extending beyond the envelope to the inside of the particle (Porter et al., 1979; Gething et al., 1980; Sleigh et al., 1980). This is consistent with the short projections seen by electron microscopy after freeze-fracture revealed the structure of the internal surface of the envelope (M. Nermut, personal communication). Little is known of the internal architecture of the virus except that what is now presumed to be M forms a layer beneath the envelope and surrounding the RNP (Compans & Dimmock, 1969; Apostolov & Flewett, 1969; Reginster
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& Nermut, 1976). Small amounts of M are found on electrophoretically purified virion RNPs in amounts independent of the size of the RNP (Rees & Dimmock, 1981). Thus, it might be tentatively suggested that the C terminus of HA2 contacts the transcriptase complex via the M protein in a manner analogous to the interaction of Semliki Forest or vesicular stomatitis virus envelope proteins with their internal components (Garoff & Simons, 1974; Dubrovi & Wagner, 1977; Mudd & Swanson, 1978). The existence of independently transcribing genome segments need not be a problem since, at an m.o.i. of 1, infectivity should be abolished if one of these failed to transcribe as the result of attachment of neutralizing antibody. However, there are data which suggest that RNP segments in virions form a continuous structure (Hoyle et al., 1961; Apostolov & Flewett, 1965; Almeida & Brand, 1975; Murti et al., 1980); therefore, criticism along these lines may have no substance.

The recent finding of antibody which binds to HA but does not neutralize infectivity (V. S. Hinshaw, personal communication) shows just how specific is the action of neutralizing antibody. We suggest that only combination of antibody with certain sites on the HA molecule can trigger the conformational changes which lead to the loss of transcriptase activity and hence of infectivity. It is also intriguing that α-NA did not modulate transcription (Fig. 4b) since NA is an integral (Drzeniek et al., 1966) and possibly a transmembrane protein (Fields et al., 1981). However, Fields et al. (1981) suggest that the NA of A/PR/8/34 is inserted into the virus envelope in the opposite orientation to HA with its NH₂ terminus projecting into the particle. Either the difference in orientation or the fact that only six residues project [compared with 11 of HA (Porter et al., 1979)] may explain why the NA does not make contact with the transcriptase complex. Alternatively, α-NA antibody may not cause conformational changes in the structure of the NA molecule.

While the hypothesis which we have advanced to explain virus neutralization is new there are precedents in the literature both for antibodies causing conformational changes in proteins and for these to be transmitted across membranes: antibody to native β-D-galactosidase from Escherichia coli enhanced activity of the defective enzyme extracted from mutant cells (Rotman & Celada, 1968), antibody to native catalase restored activity to a defective enzyme obtained from mutant mice (Fernstein et al., 1971) and the activity and normal electrophoretic behaviour of 1-amino acid oxidase was restored with antibody to the native enzyme (Zimmerman et al., 1971). An excellent example of antibody-mediated conformational changes being transmitted across membranes is implicit in the phenomenon of antigenic modulation observed when the synthesis of certain measles virus proteins was inhibited by the addition of measles virus antibodies to infected cells (Fujinami & Oldstone, 1980). Another example is the well-known mitogenic activity of antibodies directed against surface antigens of lymphocytes. While it would be premature to generate a universal hypothesis to explain neutralization of enveloped and non-enveloped, negative and positive genome viruses, there is enough evidence with poliovirus (Mandel, 1976) and bovine enterovirus (Carthew, 1976) and our own work to suggest that neutralization results from conformational changes brought about by the binding of certain determinants on the surface of a virus particle with neutralizing antibody.

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