Antibody-neutralized Avian Infectious Bronchitis Virus in Chicken Embryo Kidney Cells: Entry and Degradation

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SUMMARY

The interaction of avian infectious bronchitis virus with the chicken embryo kidney cell was studied before and after neutralization by homotypic antiserum containing only 7s (IgG) antibody. Entry of virus into the cell, measured by the ability of antibody to neutralize only extracellular virus, occurred at 37° and 25° but not at 4°. To study entry of neutralized virus, infectious bronchitis virus was adsorbed and then neutralized at 4°. The cells were incubated at 4° (control), 25°, or 37°. Antibody was dissociated from extracellular neutralized infectious bronchitis virus with a pH 2.0 buffer solution which subsequently reactivated the virus. This acid treatment for 10 sec. did not dissociate the virus from the cell or injure the cell. Antibody did not enhance or suppress elution of infectious bronchitis virus from the cell. Neutralized infectious bronchitis virus either merged with the cytoplasmic membrane or was pinocytosed at 37° and 25°; however, the neutralized virus remained extracellular at 44°. After the neutralized virus had interacted with the cytoplasmic membrane, it was not readily accessible to acid and could not be reactivated. The entry of virus without antibody was faster than that for virus with antibody. After 1 hr at 37°, the % uncoated virus RNA in cells with unneutralized virus remained essentially constant. However, in cells with neutralized virus the % uncoated virus RNA decreased and the acid-soluble material increased simultaneously.

INTRODUCTION

The role of neutralizing antibody in blocking virus infection depends on whether virus is neutralized before or after adsorption into the cell. In the former, it is generally accepted that antibody interferes with adsorption and entry of virus. Adsorption may occur, but the majority of neutralized virus does not enter the cell (Rubin & Franklin, 1957; Mandel, 1962; Joklik, 1964; Morgan, Rose & Mednis, 1968). However, even though a large number of vaccinia-antibody complexes remain at the cell surface, some virus-antibody complexes are engulfed by the host cell (Dales & Kajioka, 1964). In contrast, if antibody is added after adsorption of poliovirus, it does not prevent the virus from entering the cell (Mandel, 1962; Mandel, 1967a).

After adsorption of virus, the effect of antibody on the interaction of virus with the host cell is variable. Antibody stimulates elution of Newcastle disease virus from HeLa cells (Silverstein & Marcus, 1964) but suppresses elution of poliovirus from the same cell (Mandel, 1967a, b). Intracellular rabbitpox virus with antibody attached is only partially uncoated

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and the virus nucleic acid is not released (Joklik, 1964) but neutralized vaccinia virus (Dales & Kajioka, 1964) and neutralized poliovirus (Mandel, 1967b) are uncoated with release and subsequent degradation of the virus nucleic acid. When heterotypic antibody is added after adsorption of influenza virus, it blocks digestion of the virus envelope at the cytoplasmic membrane. Nevertheless, the virus-antibody complexes are pinocytosed in toto by the host cell (Morgan & Rose, 1968).

The present report describes the entry of both unneutralized and neutralized avian bronchitis virus into chicken embryo kidney cells. The effect of temperature and antibody on the entry and subsequent interaction of virus with the host cell was investigated.

METHODS

**Virus.** The **beaudette** strain (113th passage) of avian infectious bronchitis virus (IBV-42) was used because the plaque assay method was accurate and reproducible for this strain (Cunningham & Spring, 1965). Aggregates of virus were removed with a 200 nm filter (Wallis & Melnick, 1967). The virus is not inactivated at 37° for 120 min. in phosphate-buffered saline containing 3 % newborn calf serum (PBSS), pH 7·3. The virus is stabilized to thermal inactivation by anions (Hopkins, 1967) and is pH-stable (Stinski & Cunningham, 1969).

**IgG neutralizing antibody.** To study the interaction of neutralized IBV with the chicken embryo kidney cell (CEKC), a biologically homotypic antiserum with a high concentration of 7s (IgG) neutralizing antibody was prepared. Homotypic antibody reacts only with the surface projections of the IBV particle and does not react with the envelope (Berry & Almeida, 1968). An antiserum relatively free of 19s (IgM) antibody was necessary since IgM antibody would cause more steric hindrance than IgG. Eight-month-old Single Comb White Leghorn cockerels were inoculated intranasally with 0·2 ml., 1·6 × 10⁶ embryo 50 % infective doses (EID 50), of the **massachusetts** strain of the virus (IBV-41). Six weeks later the chickens were reinoculated with 0·1 ml., 0·8 × 10⁶ (EID 50) of the same virus. Three weeks after the second inoculation, the chickens were fasted for 24 hr and then bled. Sera were pooled and incubated at 56° for 30 min. and then stored at -20° until tested for IgM and IgG neutralizing activity. To prove that IgM neutralizing antibody was not present in the above antisera, 19s and 7s globulins were separated by centrifugation at 100,000g for 16 hr through a linear sucrose gradient (10 to 37 % sucrose in 0·15M-NaCl) in the SW39 L Spinco rotor. Before centrifugation, 0·1 ml. of whole serum, diluted two-fold in 0·85 % NaCl solution, was layered on to the gradient. Human haemoglobin (4·2s) was used as a sedimentation marker. The entire gradient was slowly moved upward through a ½ in. hole in the centre of a tightly fitted Lucite (Chromatronix, Inc., Berkeley, California) block by means of a Polystatic pump attached to a needle in the bottom of the tube. Teflon tubing of ½ in. diameter was attached to the top of the Lucite block with Chromatronix Cheminert fittings (Chromatronix, Inc., Berkeley, California). The location of neutralizing antibody in the gradient fractions was determined by the plaque reduction method (Cunningham, 1966) and the location of human haemoglobin by absorbence at 412 nm. Sedimentation coefficients were estimated by the method of Martin & Ames (1961).

Whole anti-IBV chicken serum was devoid of IgM neutralizing antibody but had a high concentration of IgG neutralizing antibody, as determined by centrifugation through a linear sucrose gradient (Fig. 1). No IgM globulins were detected by polyacrylamide gel electrophoresis. This antiserum was used for subsequent experiments.

**Cell culture.** Primary chicken embryo kidney cell (CEKC) cultures were prepared from
Neutralized IBV in CEKC

17- to 18-day-old embryos (Cunningham & Spring, 1965) and were grown in 60 mm. Falcon plastic Petri dishes using medium 199 supplemented with basal medium Eagle (BME) vitamins, BME amino acids, 5% newborn-calf serum, and with 100 units/ml of penicillin, 100 μg./ml. of streptomycin, and 50 units/ml of mycostatin.

Virus assay. Four Petri dish cultures of CEKC 48 hr old were inoculated with a 0.5 ml. suspension of virus for each sample tested. Adsorption was at 37° for 90 min., unless indicated otherwise. After adsorption, the inoculum was replaced with 4 ml. of supplemented medium 199 containing 0.9% Noble agar. The cells were incubated at 37° in 85% relative humidity and 8% CO₂ for 3 to 4 days and then 0.5 ml. of 0.1% neutral red stain was added. The plaques were counted after 30 min. at 37° and 1 hr at 4°. Virus infectivities are expressed as plaque forming units (p.f.u.) per ml. of inoculum.

Standard incubation procedure for adsorption and neutralization of virus. After adsorption of virus for 30 min. at 4°, the CEKC were washed with ice-cold PBSS to remove unadsorbed virus. Whole anti-IBV chicken serum devoid of IgM neutralizing antibody at a concentration that would neutralize approximately 90% of the adsorbed virus was added. After 30 min. at 4° the cells were washed with ice-cold PBSS to remove unreacted and unadsorbed antibody. This stage of the procedure was defined as time zero for the various experiments. Cell cultures were then incubated at either 37°, 25° or 4° (control). Adsorption and neutralization was at 4°, because virus does not enter cells at this temperature (Mandel, 1967b; Hahon & Cooke, 1967). The above incubations for adsorption and neutralization of virus at 4° are referred to as the standard incubation.

Dissociation of antibody from virus. After the standard incubation, neutralized virus was reactivated at 15 min. intervals by washing the cells for 10 sec. with glycine+HCl buffer solution (0.1 N), pH 2.0, followed immediately by two washings with PBSS. As a control, unneutralized virus was treated similarly. A neutralized virus control was washed 3 times with PBSS only. All buffer solutions were ice-cold.

Fig. 1. IgG neutralizing antibody in anti-IBV chicken serum. ●●, Neutralizing antibody: ○○, human haemoglobin. V₀, initial infectivity (3.5 x 10⁴ p.f.u./ml); V, surviving infectivity.
Horse anti-chicken globulin serum. Horse anti-chicken globulin serum was purchased from the Roboz Surgical Instrument Co., Inc., Washington, D.C.

\[^{32}P\]- and \[^{3}H\]uridine labelling of IBV. In preparation for \[^{32}P\] (Tracerlab, Waltham, Mass.) incorporation, CEKC cultures 24 hr old in medium 199, supplemented as described above, were washed with phosphate-free Hanks's (PFH) solution at pH 7.0. Citrate, 0.001 M, was substituted for the phosphates in Hanks's balanced salt solution and the pH was adjusted with 1.4% NaHCO\(_3\). To remove residual phosphates, the cells were incubated at 37\(^\circ\) for 24 hr in phosphate-free medium 199 (Grand Island Biological Co., Grand Island, New York, U.S.A.). During this period the phosphate-free medium was replaced twice. The stock virus (IBV-42) was dialysed for 72 hr against PFH and then 2 x 10\(^7\) p.f.u./ml. were added to the cells. After incubation at 37\(^\circ\) for 90 min. the inoculum was removed. Phosphate-free medium supplemented with 5% newborn calf serum dialysed for 72 hr against PFH and with \[^{32}P\] as carrier-free orthophosphate, 20 \(\mu\)c/ml., was added to the cells.

To label the RNA of the virus, CEKC were infected as previously described and supplemented medium 199 containing 30 \(\mu\)c/ml. of \[^{3}H\]uridine (3.22c/mm; Tracerlab, Waltham, Mass.) was added.

Infected cell cultures were incubated at 37\(^\circ\) for approximately 72 hr, then frozen and thawed 3 times in the medium. The mixture of medium and cell lysate was collected, centrifuged at 10,000g for 30 min., and then the supernatant fluid was collected and centrifuged at 78,000g for 2 hr. The sediment was washed, resuspended to original volume in 0.02M-phosphate buffer, pH 7.2, and subjected to a second cycle of low- and high-speed centrifugation. Sediments were washed and suspended to 1/10 the original volume with 0.02M-phosphate buffer, pH 7.2. Since the density of IBV in sucrose is similar to the density of the host cell membranes, an attempt was made to use DEAE cellulose chromatography instead of sucrose density gradient centrifugation for further purification. A 2 ml. suspension of virus was placed on a DEAE cellulose column (1 x 10 cm.) and the column was washed with 0.02M-phosphate buffer until the radioactivity in the effluent was reduced to background count. Virus was eluted from the column with 0.45M-NaCl in 0.02M-phosphate buffer, pH 7.2. Three per cent. newborn calf serum was added to stabilize infectivity. The samples were dialysed against PFH for 72 hr. Fractions with 1 x 10\(^8\) p.f.u./ml. or greater were pooled and, when necessary, concentrated by dialysis against polyethylene glycol until the radioactivity was 1 x 10\(^4\) counts/min./ml., or greater. After concentration the virus preparation was dialysed against phosphate-buffered saline solution free of magnesium and calcium (PBS), pH 7.5.

Virus labelled with \[^{3}H\]uridine was treated with RNase (Calbiochem, Los Angeles, California), 10 \(\mu\)g./ml., for 15 min. at 37\(^\circ\) and then centrifuged at 100,000g for 2 hr on to a 1 ml. cushion of 40% sucrose. The virus was resuspended to 1/10 the original volume with PBS. The virus was dialysed for 72 hr against distilled water.

Although 99% of the extraneous radioactivity was removed by centrifugation, 63% of the original infective virus was recovered. While washing the DEAE column before elution of the virus, some infective virus was removed and a large amount of virus aggregates remained on the column after elution. Consequently, the yield of infective virus was low and only 0.6% of the original radioactivity was recovered.

When a mixture of labelled non-infected CEKC medium, cell lysate and non-labelled virus were subjected to the above purification procedure, little radioactivity was associated with the virus.

The distribution of \[^{32}P\] in the virus lipid, lipoprotein and non-lipid fractions, as deter-
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mained by ether extraction (Hoyle, Horne & Waterson, 1962) was 5%, 42% and 53% respectively.

At 4° and 37° the kinetics of 32P-labelled IBV adsorption, whether measured by infectivity or radioactivity, were identical.

Radiochemical procedures. Equal volumes of extracellular PBS or infected cell lysate and RNase, 20 μg./ml. in Tris buffer (0.1 M), pH 7.5, or the above samples and Tris buffer were mixed and incubated at 37° for 15 min. To determine the acid-soluble radioactivity, crystalline bovine albumin and then trichloroacetic acid were added to each sample to a final concentration of 0.2% and 10%, respectively. The reaction mixture was held at 4° for 2 hr and then the precipitate was sedimented by centrifugation at 2200g for 1 hr. A sample of 0.5 ml. of the supernatant fluid was removed and the precipitate was resuspended in the remaining supernatant fluid, which was adjusted to pH 7.0 with 1N-NaOH. Radioactivity in the acid-soluble fraction or in the acid-soluble and acid-insoluble fraction was measured in a Mark I Nuclear Chicago liquid scintillation counter using the solvent system described by Bray (1960). Quenching of [3H]uridine activity was determined by using an external standard channels ratio. The % acid-soluble activity was then calculated. Acid-soluble radioactivity before RNase treatment was considered to be that of degraded virus material. After RNase treatment, radioactivity that was acid-insoluble was considered to be that of whole virus, and the increase in acid-soluble activity due to RNase was attributed to uncoated virus RNA.

Fig. 2. Adsorption of IBV to CEKC and the effect of acid treatment on the infectivity of adsorbed virus. ○ --- ○, 0.1 N-glycine HCl buffer solution, pH 2.0; •---•, PBS, pH 7.3.
RESULTS

Adsorption of IBV to CEKC and the effect of acid treatment on the infectivity of adsorbed virus

To determine the effect of acid treatment on IBV adsorbed into the cell, CEKC were inoculated with 150 p.f.u./ml. of virus and incubated at 37°. At various intervals the cells were washed with PBSS and then treated with acid as described. The controls were washed 3 times with PBSS only. Cells were then overlayed with agar medium, incubated and stained.

Adsorption was rapid, in that 50% of the inoculum adsorbed in 5 min. Attachment of IBV to the cells was such that brief acid treatment failed to release virus (Fig. 2). In a similar experiment at 4°, less adsorption occurred and approximately 6% of the virus was released from the cell.

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Entry of virus into CEKC

After adsorption of virus at 4°, groups of CEKC were washed with PBSS and incubated at 4° (control), 25°, or 37°. To neutralize extracellular virus at various intervals, anti-IBV chicken serum was added and the cells were incubated at 4° for 30 min. After neutralization the cells were washed with PBSS and overlayed with agar medium.

Entry of virus proceeded linearly at 37° and 25° but the virus remained sensitive to neutralization at 4° and was therefore extracellular (Fig. 3). After 45 min. at 37° the entry...
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was complete, since the subsequent addition of antibody had no effect. At 25° approximately 75% of the virus entered the cells within 120 min. (Fig. 3).

The entry rate constants (k) calculated from the relationship \( k = \ln (V_0/V_t)/t \), where \( V_0 \) is the input concentration of virus and \( V_t \) the extracellular concentration at time \( t \), were 15.87 min.\(^{-1} \), 4.49 min.\(^{-1} \), and 0.03 min.\(^{-1} \) at 37°, 25° and 4°, respectively. The same number of cells was used for each experiment at the different temperatures. The Arrhenius plot of these data indicated a linear relationship between the logarithm of the entry rate constant and the reciprocal of the absolute temperature of reaction. The energy of activation calculated from these data was approximately 19 kcal./mole.

![Graph showing the interaction of IBV-antibody complex with CEKC](image)

Fig. 4. Interaction of the IBV-antibody complex with CEKC. Entry at 37° (a), 25° (b), and 4° (c). Δ—Δ, Cells with unneutralized virus; ●—●, cells with neutralized virus, ○—○, Neutralized virus control treated with PBSS, pH 7.3. The mean and 95% confidence interval (4 determinations) are indicated for each point.
Entry of neutralized virus

After standard incubation, the proportion of neutralized virus that could be reactivated by acid treatment decreased at 37° and 25° (Fig. 4a, b). After 120 min. there was significant (5% level) reactivation at 25° but not at 37°. In contrast, the extent of reactivation at 4° was constant (Fig. 4c). The virus infectivity after acid reactivation of neutralized virus decreased approximately tenfold within 120 min. at 37° (Fig. 4a). No neutralized virus was detected in the extracellular fluid. These results suggest that neutralized IBV either merges with the cytoplasmic membrane or is pinocytosed by the host cell at 25° and 37° but not at 4°. Brief acid treatment was without effect on the cells, as determined by trypan blue, and did not inactivate the unneutralized virus control. Virus was not inactivated by exposure to 37° or to ice-cold glycine-HCl buffer solution, pH 2.0 (Fig. 4a, b, c). No increase in infectivity occurred when cells with neutralized virus were washed 3 times with PBSS only. The slight decrease in infectivity was probably due to further neutralization. The recoveries of infectivity in these experiments (data of Fig. 4a, b, c) are presented in Table 1.

Table 1. Effect of temperature and time on the recovery of infectivity by acid dissociation of antibody from IBV-cell complexes

<table>
<thead>
<tr>
<th>Temperature</th>
<th>0 min.†</th>
<th>60 min.</th>
<th>120 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°</td>
<td>54 ± 9†</td>
<td>25 ± 3</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>25°</td>
<td>48 ± 5</td>
<td>23 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>4°</td>
<td>55 ± 11</td>
<td>59 ± 5</td>
<td>56 ± 8</td>
</tr>
</tbody>
</table>

* The p.f.u./ml. recovered at various times in cells with unneutralized virus is taken as 100%.
† Immediately after the standard incubation for adsorption of antibody, the neutralized virus was reactivated by acid treatment. This is defined as time zero.
‡ ±, limits of 95% confidence interval.

The rate constants k for neutralized virus, calculated as before, were 0.12 min.⁻¹, 0.09 min.⁻¹ and 0.02 min.⁻¹ at 37°, 25° and 4°, respectively. The entry of the virus-antibody complex was also temperature-dependent, but there was a 100-fold decrease in the rate of entry as compared with that of virus without antibody. The energy of activation calculated from these data was approximately 4 kcal./mole.

Inability of horse anti-chicken globulin antibody to reneutralize cell-adsorbed virus reactivated by acid

Neutralized poliovirus reactivated by treatment with papain or at pH 4.0 retained some antibody (Keller, 1968). To determine whether chicken globulins were still associated with IBV after exposure of neutralized virus to pH 2.0, CEKC were inoculated with 120 p.f.u./ml. Adsorption of virus to cells, neutralization and acid reactivation of virus were as described. Cultures were then divided into three groups to which were added either PBSS, normal horse serum, or horse anti-chicken globulin antibody. After 30 min. at 4°, the cultures were washed with PBSS and overlayed with agar medium. Anti-chicken globulin antibody slightly increased the extent of neutralization of cell-bound virus. Approximately 42% of the neutralized virus was reactivated by acid treatment. Since this reactivated virus was not reneutralized by horse anti-chicken globulin antibody, it was concluded that treatment with a pH 2.0 buffer solution completely dissociated antibody from the virus. Equal %
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reactivation and % reneutralization were obtained with control cultures to which either PBSS or normal horse serum was added (Table 2).

Effect of temperature on the distribution of $^{32}$P in CEKC with unneutralized and neutralized $^{32}$P-labelled IBV

Using approximately $1 \times 10^4$ p.f.u./ml. of $^{32}$P-labelled IBV ($1 \times 10^5$ counts/min./ml.), adsorption of virus and then neutralization were according to the standard incubation. Cells with unneutralized (control) and neutralized $^{32}$P-labelled IBV were washed 3 times with PBS to remove unadsorbed virus and then 1 ml. of PBS was added. Groups of CEKC

![Graphs showing the effect of temperature on the distribution of $^{32}$P for CEKC with unneutralized and neutralized $^{32}$P-labelled IBV at 37°C (a), 25°C (b), and 4°C (c).](image)

Fig. 5. Effect of temperature on the distribution of $^{32}$P. Amount of radioactivity in the extracellular PBS (---) and that associated with the cell (----) for CEKC with unneutralized (△, ◊) or neutralized (●, ◌) $^{32}$P-labelled IBV. Groups of CEKC incubated at 37°C (a), 25°C (b), and 4°C (c).
cultures were incubated at 37°, 25°, or 4° (control). At 15 min. intervals the extracellular PBS was removed and the cells were scraped off the Petri dish and suspended in 0.5 ml. of PBS. The 32P radioactivity in the extracellular PBS and associated with the cells was determined.

Table 2. Inability of horse anti-chicken globulin antibody to reneutralize cell-adsorbed virus reactivated by acid dissociation of antibody from virus

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Initial neutralization (%)</th>
<th>Reactivation by exposing neutralized cell-adsorbed virus to pH 2.0 buffer solution for 10 sec.*</th>
<th>Plaque reduction by reneutralization with anti-chicken globulin antibody (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Neutralized virus + PBSS</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Neutralized virus + normal horse serum</td>
<td>93.8</td>
<td>87.3</td>
<td>43.3</td>
</tr>
<tr>
<td>Neutralized virus + horse anti-chicken globulin antibody</td>
<td>88.8</td>
<td>86.1</td>
<td>46.8</td>
</tr>
</tbody>
</table>

* The unneutralized virus controls, not affected by these treatments, is taken as 100%.
† Anti-IBV chicken serum was diluted 1/20 and 1/30 for experiments 1 and 2, respectively.

Table 3. Elution of infective virus, 32P, and degraded virus material from CEKC with 32P-labelled IBV, unneutralized and neutralized

<table>
<thead>
<tr>
<th>Minutes at 37°</th>
<th>Infectivity eluted (%)</th>
<th>32P eluted from CEKC (%)</th>
<th>[32P] TCA-soluble (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unneutralized virus*</td>
<td>Neutralized virus</td>
<td>Unneutralized virus*</td>
</tr>
<tr>
<td>0</td>
<td>0.7</td>
<td>1.8</td>
<td>3.1</td>
</tr>
<tr>
<td>15</td>
<td>0.7</td>
<td>11.5</td>
<td>13.4</td>
</tr>
<tr>
<td>30</td>
<td>0.8</td>
<td>23.3</td>
<td>18.3</td>
</tr>
<tr>
<td>45</td>
<td>0.9</td>
<td>34.9</td>
<td>26.9</td>
</tr>
<tr>
<td>60</td>
<td>0.9</td>
<td>44.9</td>
<td>32.6</td>
</tr>
<tr>
<td>75</td>
<td>0.9</td>
<td>43.0</td>
<td>42.6</td>
</tr>
<tr>
<td>90</td>
<td>1.0</td>
<td>52.5</td>
<td>54.5</td>
</tr>
<tr>
<td>105</td>
<td>1.1</td>
<td>50.6</td>
<td>49.9</td>
</tr>
<tr>
<td>120</td>
<td>1.1</td>
<td>61.6</td>
<td>52.6</td>
</tr>
</tbody>
</table>

* Data for neutralized and unneutralized virus are not significantly different at 10% level.

At 37° the 32P activity associated with the cells decreased for 75 min. and that of the extracellular PBS increased simultaneously (Fig. 5a). In contrast, the 32P-cell-associated activity decreased slightly at 25° and there was no change in the 32P-cell-associated activity in the 4° control. At all temperatures there was no difference in the distribution of 32P for cells with neutralized and unneutralized IBV (Fig. 5a–c).

The extracellular PBS was also analysed for infectious virus, 32P, and degraded virus material. At 37° approximately 1% of the adsorbed unneutralized virus eluted from the cells (Table 3). However, it is possible that some of the adsorbed virus was no longer infective after elution. The majority of the eluted radioactivity was present in acid-insoluble virus material, although approximately a quarter of the activity was due to degraded virus material. There were no significant differences (10% level) between unneutralized or
neutralized IBV with respect to the % [32P] eluted or the % degraded virus material in the extracellular PBS (Table 3). Therefore, antibody neither enhanced nor suppressed elution of virus and did not interfere with the degradation of virus.

After 120 min. at 25°, the small amount of [32P] activity in the extracellular PBS was mainly acid-insoluble.

**Degradation of neutralized IBV in CEKC**

Using approximately $1 \times 10^4$ p.f.u./ml. of [32P]-labelled IBV ($1 \times 10^6$ counts/min./ml.), adsorption of virus and then neutralization were according to the standard incubation. The cells were washed 3 times with PBS and 1 ml. of PBS was added and then the cells were incubated at either 37°, 25°, or 4° (control). At various intervals the extracellular PBS was removed and the cells were washed with PBS, suspended in 0.5 ml. of double distilled water, and lysed by 4 cycles of freezing and thawing. The cells were then analysed for acid-insoluble RNase resistant, acid-insoluble RNase sensitive, and acid-soluble material.

Fig. 6. Interaction of unneutralized and neutralized [32P]-labelled IBV with CEKC. Cells with unneutralized (——) and neutralized (----) IBV incubated at either 37° or 4° (control). Acid-soluble, RNase resistant (▲, △), acid-insoluble, RNase sensitive (●, ○) and acid-soluble material (■, □).

There was no increase in the [32P]-acid-soluble activity in the 4° controls. However when virus was incubated at 37° for 15 min., approximately 3% of the radioactivity was in solution and subsequently there was a 5 to 6% increase in RNase-sensitive material. Similar results were obtained with [3H]uridine-labelled IBV. Therefore, the RNase-sensitive material initially detected was probably due to externally adsorbed virus damaged at 37° (Fig. 6). No cell-associated degraded virus material was detected initially.

At 37° approximately the same amount of whole virus and degraded virus material was
associated with cells containing unneutralized or neutralized virus (Fig. 6). Maximum intracellular degradation of virus did not occur until after 120 min. At 37° the amount of RNase-sensitive 32P-labelled virus RNA remained at approximately 12% in cells with unneutralized virus but decreased to 1% in cells with neutralized virus (Fig. 6). This difference in the amount of acid-insoluble RNase-sensitive material was most apparent at a time when the majority of neutralized virus was intracellular.

At 25° only 8% of degraded virus material was detected and there were no detectable differences between unneutralized and neutralized virus.

Table 4. Degradation of neutralized [3H]uridine-labelled IBV in CEKC at 37°*

<table>
<thead>
<tr>
<th>Min.</th>
<th>Unneutralized</th>
<th>Neutralized</th>
<th>Increase in cell-associated [3H]TCA soluble after RNase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>virus</td>
<td>virus</td>
<td>Unneutralized virus Neutralized virus</td>
</tr>
<tr>
<td>0</td>
<td>-3.6</td>
<td>0</td>
<td>8.5 5.9</td>
</tr>
<tr>
<td>30</td>
<td>2.9</td>
<td>1.4</td>
<td>5.3 5.1</td>
</tr>
<tr>
<td>90</td>
<td>1.5</td>
<td>13.7</td>
<td>6.2 2.0</td>
</tr>
<tr>
<td>150</td>
<td>1.8</td>
<td>9.7</td>
<td>6.6 1.7</td>
</tr>
<tr>
<td>210</td>
<td>0</td>
<td>8.5</td>
<td>7.4 1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Min.</th>
<th>Unneutralized</th>
<th>Neutralized</th>
<th>Increase in extracellular PBS [3H]TCA soluble (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>virus</td>
<td>virus</td>
<td>Unneutralized virus Neutralized virus</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.6 0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0.3</td>
<td>0.3 -2.7</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
<td>1.7</td>
<td>3.8 0</td>
</tr>
<tr>
<td>150</td>
<td>-2.5</td>
<td>3.6</td>
<td>8.4 -1.4</td>
</tr>
<tr>
<td>210</td>
<td>3.8</td>
<td>7.1</td>
<td>7.8 0.8</td>
</tr>
</tbody>
</table>

* At each time the % acid-soluble radioactivities at 4° (control) was subtracted from that at 37°.

To determine more accurately the amount of acid-insoluble material sensitive to RNase the above experiment was repeated at 37° using approximately $1 \times 10^6$ p.f.u./ml of [3H]uridine-labelled IBV ($1 \times 10^6$ counts/min./ml.). In addition, the extracellular PBS was also analysed. The amount of RNase-sensitive [3H]uridine-labelled virus RNA in cells with unneutralized virus remained constant. However, in cells with neutralized virus the amount of acid-insoluble RNase-sensitive material decreased and the acid-soluble material increased simultaneously (Table 4). The extracellular PBS from cells with unneutralized virus contained 4 to 8% acid-insoluble RNase-sensitive material. However, none of this material was detected in the extracellular PBS from cells with neutralized virus (Table 4).

**DISCUSSION**

Although many viruses, including infectious bronchitis virus, attach to cells at 4°, they do not enter the cells and are consequently vulnerable to antibody (Mandel, 1962; Homma & Graham, 1965; Mandel, 1967; Morgan & Rose, 1968). However, when viruses enter cells at 25° to 37°, they are invulnerable to antibody (Hahon & Cooke, 1967; Mandel, 1967).
Neutralized IBV in CEKC

It was possible to detect neutralized IBV at the cell surface by dissociating antibody from the complex by acid treatment. The reactivated virus produced plaques in cultures of the cells to which they had been adsorbed. Acid treatment of chick embryo kidney cells did not release IBV from the cells, inactivate the viability of the cells, or interfere with the subsequent replication of IBV. The inability to detect chicken globulins attached to cell-adsorbed virus after exposure to a pH 2.0 buffer solution indicated that antibody was dissociated completely from virus. Even though neutralized poliovirus was reactivated by papain or exposure to pH 4.0, antibody was still associated with the virus (Keller, 1968).

Since reactivation of neutralized IBV decreased with time at 37° and 25°, neutralized IBV was either entering the cells or eluting from the cells. Antibody did not enhance elution of IBV from CEKC, therefore neutralized IBV must have entered the cells. If neutralized virus merged with the cytoplasmic membrane or was pinocytosed by the cell, it could not be reactivated because the antibody would no longer be readily accessible to the acid. Since no entry of IBV occurred at 4°, it is probable that the process of entry may require enzymic activity which is inhibited at 4°.

Alternative hypotheses for the decrease in acid reactivation of neutralized virus are, that a stable bond is formed between antibody and virus, or that a number of antibody molecules which react with virus increases with time and subsequently prevents reactivation. These interpretations are inadequate because the majority of IBV neutralized in vitro by long incubation with high concentrations of antibody can be reactivated by pH 2.0 (Stinski & Cunningham, 1969).

The rate of entry of poliovirus into HeLa cells at 37° is independent of the presence of antibody (Mandel, 1962; Mandel, 1967a). The entry of neutralized poliovirus at 37° into cells began immediately, was exponential for 30 min., then progressed less rapidly. Although the entry of neutralized IBV at 37° also began immediately, the entry rate constant was lower than that for unneutralized virus and the reaction was first order for the 120 min. of the experiment. It is possible that the mechanism of IBV entry into the cell is influenced by the attachment of antibody.

The mechanisms by which unneutralized enveloped viruses such as herpes simplex (Morgan et al. 1968), influenza (Morgan & Rose, 1968), and Sendai (Morgan & Howe, 1968) enter the cell involve digestion of the virus envelope and of the host cell cytoplasmic membrane at the point of contact. However, Dales (1969) reported that the entry of herpes simplex virus was by pinocytosis and was not due to digestion at the point of contact. With influenza virus, the addition of heterotypic antibody after adsorption of virus blocks digestion, although the virus-antibody complexes are pinocytosed in toto by the host cell (Morgan & Rose, 1968). The entry of IBV without antibody may be by digestion of the envelope at the cytoplasmic membrane and that of the IBV-antibody complex by pinocytosis.

Although, there were no significant differences in the accumulation of degraded 32P-labelled virus material in cells with unneutralized or neutralized virus, there was a difference in the amount of RNase-sensitive 32P-labelled virus RNA. In cells with neutralized [3H]uridine-labelled virus, a decrease in RNase-sensitive material occurred and the acid-soluble material increased simultaneously. However, the amount of RNase-sensitive material remained constant in cells with unneutralized virus. A decrease in the amount of intact RNA from neutralized 32P-labelled poliovirus occurs within 15 min. at 37° (Mandel, 1967b). However, experiments with 3H-labelled vaccinia DNA indicated that the virus DNA from neutralized virus is degraded within 1 to 2 hr at 37° (Dales & Kajioka, 1964).

Studies with 32P-labelled poliovirus (Mandel, 1967b) suggested that antibody may either suppress a reaction necessary for the release of intact virus RNA or stimulate a reaction which
rapidly degrades the released RNA. The latter hypothesis was considered to be more favourable. The results obtained with antibody attached to IBV indicate that the virus RNA is released and subsequently degraded. However, further studies are necessary to determine how the cell interacts within neutralized IBV. With antibody attached, IBV may be completely degraded with pinocytic vesicles by lysosomal enzymes. The RNase in pinocytic vesicles could destroy the virus RNA and consequently prevent replication.

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REFERENCES


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