The Morphological and Biological Effects of Various Antisera on Avian Infectious Bronchitis Virus

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SUMMARY

Morphologically, homotypic and heterotypic antisera neutralized avian infectious bronchitis virus significantly more when unheated. Morphologically, using the electron microscope technique of negative staining, there was a clear distinction between the effects of homotypic and heterotypic antisera. Heated homotypic antiserum revealed antibody attached only to the projections of the virus, while with unheated homotypic serum heat labile components could be visualized but no basic change could be seen in particle morphology. Heterotypic serum contained antibodies directed both against the projections and the envelope of the virus. In addition, unheated heterotypic antiserum produced holes approximately 100 Å in diameter in the virus membrane, suggesting that a form of virus lysis takes place. Rabbit antiserum prepared against uninfected chick-embryo fibroblasts was able to produce similar holes in the virus envelope and this led us to postulate that the envelope component of avian infectious bronchitis virus is closely related to normal chick host material.

INTRODUCTION

In assessing the neutralizing ability of an antiserum for a virus the serum is usually heated. This is done because the presence of heat-labile serum components can considerably enhance the neutralizing ability of the serum, thus giving an incorrect value of potency (Lennette, 1964). The present study was undertaken to investigate the biological effect on infectious bronchitis virus of heated and unheated antiserum and also to examine the possibility of visualizing by electron microscopy any difference between the appearance of a two-component antigen+antibody system (Lafferty & Oertelis, 1961; Hummeler, Anderson & Brown, 1962; Almeida, Cinader & Howatson, 1963) and a three-component antigen+antibody + complement system (Borsos, Dour Mashkin & Humphrey, 1964).

METHODS

Virus. Avian infectious bronchitis virus preparations were made from the allantoic fluid of virus-infected chick embryos (Berry et al. 1964).

Sera. Antiserum was obtained from pathogen-free fowls (homotypic serum) and rabbits (heterotypic serum) one week after the administration of the last of four weekly

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intramuscular doses of $10^6$ EID 50 of infectious bronchitis virus. Normal serum was stored at $4^\circ$ or at $-20^\circ$ and used as soon as possible after collection. Heated serum was held at $56^\circ$ for 30 min. before being used. Fowl C'1 was prepared as described by Benson, Brumfield & Pomeroy (1961).

**Electron microscopy.** Virus + serum mixtures were prepared for electron microscopy by diluting 0.1 ml. of virus with 0.5 ml. phosphate-buffered saline (pH 7.0), mixing well and then adding 0.1 ml. of serum with further mixing. Four groups of sera were studied, homotypic, heated and unheated, and heterotypic, heated and unheated. Non-immune control sera were studied alongside the specific antisera. The mixtures were incubated at $37^\circ$ for 1 hr then left at $4^\circ$ overnight. The next morning the virus + serum mixtures were centrifuged for 30 min. at 10,000 rev./min., the sediment resuspended in phosphate-buffered saline to the original volume, centrifuged as before and the pellet so obtained resuspended in approximately 0.1 ml. distilled water. This suspension was negatively stained by mixing a drop with an equal quantity of 3% phosphotungstic acid adjusted to pH 6. A small amount of this mixture was transferred to 400-mesh carbon-formvar coated grids and examined immediately in a Philips 200 electron microscope.

**Serology.** Virus + serum mixtures the same as those used for electron microscopy were incubated for 1 hr at $37^\circ$ and then left at $4^\circ$ overnight. The next morning they were assayed for surviving virus by making tenfold dilutions in saline and titrating for infectivity in chick embryos. Five embryos per dilution were used giving an error of $\pm 0.5$ log. (Page & Cunningham, 1962).

**RESULTS**

Heated homotypic and heterotypic sera neutralized duplicate samples of virus to precisely the same extent; both reduced infectivity by 2.5 log. units. Similarly, unheated homotypic and heterotypic antiserum neutralized the virus to almost the same extent, but with a reduction in infectivity of approximately 5 log. units (Tables 1, 2). Enhanced activity could be restored to heated serum in the heterotypic system by the addition of either unheated non-immune rabbit serum or guinea-pig complement and in the homotypic system by unheated non-immune fowl serum or fowl C'1 plus guinea-pig serum. The high neutralizing capacity of non-immune heated serum for avian infectious bronchitis virus is a feature of this virus and has been discussed elsewhere (Cunningham, 1951).

**Electron microscopy.** Although the serological effects of homotypic and heterotypic antisera were not very different, the electron microscopic appearances of the two serum groups were markedly different and will be discussed separately.

**Homotypic serum.** Plate 1, fig. 1, shows the appearance of negatively stained infectious bronchitis virus particles. The particles are pleomorphic, in the size range 800 to 1000 Å in diameter, and display distinctive projections on the surface (Berry et al. 1964). The projections are 200 Å long, petal-shaped in outline, and attached to the virus by an extremely narrow stalk. They seem to be easily detached from the virus and often the particles are incompletely covered by them. Besides illustrating the appearance of infectious bronchitis virus the particles in Fig. 1 serve as a control, as they had been incubated with normal fowl serum but had remained unchanged. Complexes of virus and heated fowl (homotypic) antiserum revealed the presence of
large aggregates of virus particles (Pl. 1, fig. 2). Virus particles within these aggregates were linked by small thread-like structures such as have been described in several virus antibody studies (Lafferty & Oertelis, 1961; Almeida et al. 1963). Although it is impossible by microscopy alone to identify these structures as antibody molecules the stringent use of controls (Almeida et al. 1963) makes it likely that they do indeed represent specific attachment of immune globulin. The outstanding finding with homotypic antiserum was that, while such molecules linked large rafts of virus particles together, they were attached solely to the virus projections. This gave rise to a gap between the main body of the particle and the point where antibody was attached to the viral envelope (Pl. 2, fig. 3).

Table 1. Surviving infectious virus in unheated and heated fowl serum + infectious bronchitis virus mixtures

<table>
<thead>
<tr>
<th>Virus mixed with</th>
<th>Surviving virus (log.)</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6.2</td>
</tr>
<tr>
<td>Unheated normal serum</td>
<td>4.5</td>
</tr>
<tr>
<td>Fowl C' I</td>
<td>5.0</td>
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<tr>
<td>Unheated antiserum</td>
<td>1.2</td>
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<tr>
<td>Heated antiserum</td>
<td>3.7</td>
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<tr>
<td>Heated antiserum + unheated normal serum</td>
<td>1.5</td>
</tr>
<tr>
<td>Heated antiserum + fowl C' I</td>
<td>3.7</td>
</tr>
<tr>
<td>Heated antiserum + unheated guinea-pig serum</td>
<td>3.7</td>
</tr>
<tr>
<td>Heated antiserum + fowl C' I + unheated guinea-pig serum</td>
<td>1.0</td>
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</tbody>
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Table 2. Surviving infectious virus in unheated and heated rabbit serum + infectious bronchitis virus mixtures

<table>
<thead>
<tr>
<th>Virus mixed with</th>
<th>Surviving virus (log.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6.2</td>
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<tr>
<td>Unheated normal serum</td>
<td>5.5</td>
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<tr>
<td>Unheated antiserum</td>
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<tr>
<td>Heated antiserum</td>
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<tr>
<td>Heated antiserum + unheated normal serum</td>
<td>1.7</td>
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<tr>
<td>Heated antiserum + unheated guinea-pig serum</td>
<td>1.0</td>
</tr>
<tr>
<td>Unheated rabbit anti-chick-embryo serum</td>
<td>2.7</td>
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When unheated homotypic antiserum was used to form the virus + antiserum complex, no overall change in the virus aggregates was seen. Particles were still linked by molecules attached to the projections and the basic appearance of the particles remained unchanged. However, occasional particles were surrounded by a much wider halo (Pl. 2, fig. 4). This at times measured up to 700 Å while with heated antiserum it was never more than 300 Å in diameter. The larger halo was also present when the virus was incubated with a mixture of heated antiserum and unheated normal fowl serum.

Heterotypic serum. As with the homotypic system the virus was clumped into large aggregates by heterotypic heated antiserum. However, with heterotypic serum the antibody molecules were attached not only to the virus projections but also to the virus
envelope. Spacing between the particles in aggregates was much smaller than with the homotypic system and there was no clear rim left around the particles (Pl. 2, fig. 5).

Unheated heterotypic antiserum produced a very marked change in the appearance of the particles forming the complex. Antibody molecules were attached to the projections and envelope as seen in heated rabbit-antiserum mixtures, but in addition the particles appeared flattened and the viral envelope revealed numerous holes approximately 100 Å across (Pl. 3, fig. 6, 7).

The ability to produce holes in the virus envelope was restored to heated rabbit antiserum by the addition of either unheated control rabbit serum or normal unheated guinea-pig serum (Pl. 3, fig. 8).

Heated rabbit anti-chick-embryo antibody + virus mixtures showed antibody attachment to the virus envelope only, while unheated rabbit anti-chick-embryo antiserum in addition produced holes in the virus envelope (Pl. 3, fig. 9).

DISCUSSION

Irrespective of whether homotypic or heterotypic antisera was used our results show that there is a considerable difference between the effect of a two-component antigen + antibody interaction, and a three-component antigen + antibody + complement system on infectious bronchitis virus. Combination with specific antibody alone resulted in a reduction in infectivity of 2 log. units (Tables 1, 2) while the presence of heat labile components in the unheated serum resulted in an overall reduction of 5 log. units.

However, while the homotypic and heterotypic sera acted biologically in an almost identical manner, the electron microscope revealed that the two types of sera reacted with infectious bronchitis virus in markedly different ways. Use of antiserum from the host species (homotypic) revealed molecules attached only to the projections of the virus, while rabbit (heterotypic) antiserum showed molecules attached both to the virus projections and the virus envelope. A simple explanation of this phenomenon would be that the host species, fowl, is unable to recognize the virus envelope as being antigenically different from normal host material. A heterologous species on the other hand would recognize all the viral components as foreign. This argument is substantiated by the fact that antiserum prepared in rabbits against uninfected chick embryo tissue was capable both of neutralizing the virus and, when unheated, of reacting with the virus envelope to produce holes. The fact that interaction of the virus with homotypic antiserum shows no attachment of molecules to the envelope component of the virus leads us to speculate that infectious bronchitis virus is unusual inasmuch as one of the external components, that is the envelope, is not able to elicit any immune response in the host species.

The other major distinction between the action of the homotypic and the heterotypic antiserum was that heterotypic antiserum which had not been heated produced distinctive holes in the virus envelope. These holes are indistinguishable from the ones produced in red cell membranes by the action of antibody and complement (Borsos et al. 1964), and since it is these holes in the lipoprotein component of the membrane that lead to red cell lysis (Humphrey & Dourmashkin, 1965) it is probable that the action of heterotypic antibody plus heat-labile components on infectious bronchitis virus has a similar mechanism.
Antisera and avian infectious bronchitis virus

The phenomenon of lysis would explain the enhancing effect of heat labile components in heterotypic antiserum but there is no such explanation for the enhanced neutralizing ability of unheated homotypic antiserum. Homotypic antiserum would not be expected to produce holes in the virus envelope as it does not have antibody directed against this component, and except for the wider halo visualized around occasional virus particles (Pl. 2, fig. 4) there is no morphological distinction between heated and unheated antiserum. This leads to the suggestion that heat labile components may have different modes of action depending on the nature of the antigenic structure. That is, when antibody is directed against a lipoprotein membrane heat labile components enable lysis to occur; against protein the effect is one that cannot be recognized morphologically. It should be pointed out here that in the system we have used there is a further complication introduced by the fact that we are comparing a mammalian with an avian antiserum. Further work is at present being undertaken which should overcome this difficulty.

Feinstein & Munn (1966), using a three-component system of bacterial flagellar antigen, antibody and complement, showed that the components of the complement system could be visualized by negative staining. Their micrographs bear a close resemblance to the additional halo (Pl. 2, fig. 4) surrounding occasional particles after interaction with unheated sera, and it seems reasonable that here too it is part of the complement system that is being visualized. From an electron microscopic viewpoint it becomes important then that sera to be used for morphological antibody studies should always be heated.

In conclusion the effect of unheated heterotypic antiserum on infectious bronchitis virus shows that some type of viral lysis can occur. By analogy with the red cell system this lysis is dependent on the presence of a lipoprotein membrane and viral lysis may be a simple explanation of the fact that several viruses (Lennette, 1964; Yoshino & Taniguchi, 1965, 1967) having a lipoprotein component are sensitive to the action of heat labile components in antiserum.

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REFERENCES


EXPLANATION OF PLATES

PLATE I

Fig. 1. A group of negatively stained avian infectious bronchitis virus particles that had been treated with non-immune rabbit serum. The particles remain unaltered and illustrate the morphological features of this virus.

Fig. 2. A group of virus particles corresponding to those shown in fig. 1 but which were treated with heated homotypic fowl antiserum. The particles are clumped and the surface projections are obscured.

PLATE 2

Fig. 3. A higher power of two particles from the same preparation as fig. 2.

Fig. 4. The overall appearance of infectious bronchitis virus treated with unheated fowl serum.

Fig. 5. A group of infectious bronchitis virus particles that had been mixed with heated specific rabbit (heterotypic) antiserum. This micrograph should be compared with fig. 2.

PLATE 3

Fig. 6. This micrograph shows part of a complex of infectious bronchitis virus and unheated rabbit antiserum. The particles appear flattened and distinct holes, approximately 100 Å across, appear in the virus membrane.

Fig. 7. A higher power of a virus particle treated with unheated rabbit antiserum. Here it can be seen that the holes have a distinct appearance with a definite wall around them.

Fig. 8. In this illustration the particles had been treated with a mixture of heated specific rabbit antiserum and unheated control rabbit serum. The particles display the typical 100 Å holes.

Fig. 9. A group of virus particles that had been mixed with rabbit anti-chick-embryo-fibroblast serum. The projections of the particles are clearly visible but the presence of holes in the envelope shows that the serum has exerted a lytic effect.
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Plate 2

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