Morphological studies of the neutralization of influenza virus by IgM

S. J. Armstrong, M. C. Outlaw and N. J. Dimmock*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

Quantitative relationships between neutralization, aggregation and attachment to monolayers of chick embryo fibroblast (CEF) cells have been studied using a constant amount of influenza A/fowl plague virus/Rostock/34 (H7N1) and varying amounts of purified mouse polyclonal IgM directed against the haemagglutinin, the major viral neutralization antigen. There are two major types of interaction. (i) At low concentrations of IgM there is aggregation of virus, but no neutralization provided that the aggregates are dispersed by vortexing and dilution. Maximum aggregation occurs at less than seven molecules of IgM per virion and the IgM is probably bound in the ‘staple’ or ‘crab’ conformation at these concentrations. (ii) At higher concentrations there is neutralization and this coincides with inhibition of attachment of virus to CEF cells. Neutralization of 50% infectivity requires about 35 molecules of IgM per virion. The maximum neutralization observed was only 87%. Quantitative data and electron microscopy observations suggest that molecules of IgM at the higher concentrations adopt a planar stance approximately perpendicular to the viral surface. It appears that IgM neutralizes fowl plague virus in vitro primarily by interfering with its attachment to cells; the fraction of neutralized virus that does attach is known not to be internalized.

Introduction

IgG, IgA and IgM can all cause ‘intrinsic neutralization’, the loss of infectivity which ensues when a virus particle binds antibody. Definition of the mechanism of neutralization of virus by antibody is relevant only to a particular combination of virus, the neutralization determinants of that virus, the isotype of antibody and the cell in each system being studied (reviewed by Dimmock, 1984, 1987). Historically, however, it was assumed that antibody prevented attachment of virus to a cell by steric hindrance. In fact, for IgG the opposite prevails and data from many different virus–IgG–cell systems have shown that IgG neutralization does not affect attachment. On the other hand, there are a few reports of situations where IgG neutralization does prevent attachment (reviewed by Dimmock, 1984, 1987). M. C. Outlaw et al. (unpublished observations) have shown that influenza virus saturated with IgG binds normally to baby hamster kidney (BHK) or tracheal epithelial cells, but attachment of virus neutralized at lower concentrations of antibody is partially inhibited, indicating that the relative proportions of antibody and virus are also important. In another system attachment of influenza virus to a B lymphoma cell line was inhibited by IgG (Eisenlohr et al., 1987).

As well as attaching to cells, IgG-neutralized influenza virus appears to be internalized normally (Possee et al., 1982). Infectious virus enters the cytoplasm by endocytosis (reviewed by Marsh, 1984; Patterson & Oxford, 1986) and the virus core moves to the nucleus, where transcription takes place. Neutralized influenza is also uncoated and its core migrates to the nucleus but no transcription ensues (Possee et al., 1982; Rigg et al., 1989). The precise mechanism of neutralization has not yet been elucidated but there is evidence that uncoating of the core, which is a necessary prerequisite for transcription, is prevented (Rigg et al., 1989).

Secretory (s) IgA has a different mechanism of neutralization. Each IgA antibody is a dimer or higher multimer, linked by the Fc regions with the antigen-binding oriented in opposite directions. When interacting with influenza virus it can span two or three haemagglutinin (HA) spikes, whilst binding to further spikes at each end of the ‘bridge’. High concentrations of monoclonal polymeric IgA and of polyclonal slgA impede attachment to the cell and/or internalization of those virions which do attach (Taylor et al., 1985a; Outlaw & Dimmock, 1990), but IgA monomers appear to act in the same way as IgG (Taylor & Dimmock, 1985a). Recent observations suggest that low neutralizing concentrations of IgA permit attachment and internalization but probably prevent the fusion of the virus with the endocytic vesicle by cross-linking surface spikes (S. J. Armstrong & N. J. Dimmock, unpublished data).

IgM is composed of five subunits forming a flat,
starfish-shaped molecule of roughly 30 nm in diameter (Feinstein et al., 1971). At a single high concentration which neutralized over 90%, IgM prevented attachment of influenza virus to BHK cells at 4 °C and of about half the virus at 25 °C or 30 °C and entry of attached virus did not take place (Taylor & Dimmock, 1985b). Outlaw & Dimmock (1990) have confirmed these data using primary mouse tracheal epithelial cells and mouse IgM. Similarly, IgM reduced attachment of neutralized poliovirus to HeLa cells (Mandel, 1967).

This paper describes some quantitative morphological studies on the mechanism of neutralization by IgM. These permit us to conclude that it is the number of IgM molecules per virion which determines how the virus–antibody complex behaves with regard to intrinsic neutralization, aggregation of virus particles and ability to attach to cells.

**Methods**

**Virus.** The avian strains of influenza A/fowl plague virus/Rostock/34 (H7N1) (FPV/R) and A/fowl plague virus/Dutch/27 (H7N7) (FPV/D), were grown in 11-day-old embryonated hen's eggs, purified (Kelly & Dimmock, 1974) and stored at -70 °C.

The stock FPV/R virus preparation contained 1-1 × 10⁶ haemagglutination units (HAU) and 2.4 × 10¹¹ p.f.u. per ml. It was used diluted 1/500 in phosphate-buffered saline (PBS).

**Antibody.** Polyclonal IgM was raised in adult C3H/He-Mg mice by a single intravenous injection of 10⁴ HAU of β-propiolactone (BPL)-inactivated FPV/D (Barrett et al., 1984). Polyclonal IgM was purified from serum taken 6 days later by Sephacryl-300 chromatography. Its identity was confirmed by double immunodiffusion against anti-mouse IgM and anti-mouse IgG and it was the only protein detected by PAGE (data not shown).

Monoclonal IgG to the HA (HC2) was purified from mouse ascitic fluid on a Protein A-Sepharose column.

**Infectivity assay.** FPV/R was plaque-assayed on primary chick embryo (CEF) cell monolayers (Morser et al., 1973). Where appropriate the virus was incubated for 1 h at room temperature with equal volumes of purified IgM at different concentrations. Samples were taken for electron microscopy and the preparations were aspirated vigorously and vortexed to break up aggregates before assaying for infectivity.

**Electron microscopy (EM)**

(i) **Aggregation of virus by IgM.** Samples of virus and IgM were incubated together at room temperature. They were then prepared for EM by mixing with an equal volume of 3% aqueous sodium silicotungstate (SST) pH 6.5, put on Formvar-coated grids and examined with a Jeol 100C transmission electron microscope. Counts were made of the numbers of single virions and those in aggregates of two to five, six to 20 and > 20 virions. In control preparations roughly half the virions were single and the rest were in aggregates of two to five.

(ii) **Assay for the presence of IgM.** Five µl samples of virus previously treated with IgM for 1 h at room temperature were adsorbed onto Formvar-coated grids, washed with PBS, then treated for 30 min at room temperature with 5 µl goat anti-mouse IgM tagged with 10 nm particles of gold colloid (E. Y. Labs). The grids were washed and stained with SST. The number of grains over individual virions (n = 100) was counted and compared with the control (n = 350) and the results were analysed using Student's t-test.

(iii) **Attachment of virus to cells.** Virus and IgM or PBS were incubated together for 1 h at room temperature and inoculated at 4 °C for 1 h onto CEF cell monolayers grown in small plastic flasks at an m.o.i. equivalent to 100 p.f.u./cell. Cells were pre-fixed with 0.2% glutaraldehyde for 15 min at 4 °C followed by 0.5M-ammonium chloride for 15 min. Virus particles attached to cells were further tagged with anti-HA monoclonal IgG (HC2) overnight at 4 °C. The cell sheets were washed and incubated with a mixture of gold-labelled anti-mouse IgG and anti-mouse IgM (E. Y. Labs), both diluted in PBS with 0.1% bovine serum albumin (BSA), for 1 h at room temperature.

The cells were processed for electron microscopy using standard methods. Surface virions were gold-labelled either by reaction with HC2 bound to HA spikes or to the IgM (if present) on the surface and were easily identified. The number of virions on each of 50 cells was counted for each sample.

**Iodination of IgM.** ¹²⁵I-labelled IgM was prepared by the chloramine-T method (Hunter & Greenwood, 1962). Free radiolabel was separated by G-25 Sephadex chromatography (Pharmacia) and fractions containing peak haemagglutination inhibition activity and radioactivity were pooled. BSA was added to 1% (w/v) and purified IgM stored at 4 °C.

Various dilutions of IgM were incubated with FPV/R and the amount of bound radioactivity was determined after separating the unbound IgM by centrifugation through 5% sucrose. Influenza B/Lee virus was used to monitor non-specific binding. The number of IgM molecules per virion was calculated from the specific activity of the IgM (3 × 10⁶ c.p.m./µg), the number of virions per HAU (3.98 × 10⁷; Taylor et al., 1987) and Avogadro's constant.

**Results**

**Neutralization of FPV/R by IgM**

Fig. 1(a) shows a plaque inhibition assay demonstrating the effect of doubling dilutions of IgM on the infectivity of FPV. Samples were vortexed before assay to break up viral aggregates which would otherwise reduce infectivity. This preparation of IgM gave a maximum of 87% neutralization. There was no neutralization at a dilution of 1/32.

**Aggregation of FPV/R by IgM**

FPV treated with serial dilutions of IgM was negatively stained and examined under the electron microscope. Several hundred virions were examined and the numbers which were single or in groups were noted. The results were expressed as the percentage of the total number of virions which were single or fell into three categories of aggregate size. Fig. 1(b) shows the extent of aggregation of FPV/R as a function of antibody concentration. At high concentrations of IgM antibody was visible as a coating on the virions. Here the distribution was similar to the controls; there was no cross-linking between particles presumably owing to the lack of available
Neutralization by IgM

Fig. 1. Comparison of (a) neutralization and (b) aggregation of FPV/R by IgM. FPV/R was incubated with dilutions of IgM for 1 h at room temperature. Samples were mixed with negative stain, and put onto grids for EM. Corresponding portions were vortexed to disaggregate the virus and were plaque-assayed on CEF cells to assess neutralization. Panel (b) shows the number of particles per aggregate in the undiluted, non-vortexed samples and demonstrates that IgM is detectable by aggregation of virus at dilutions of antibody which do not intrinsically neutralize individual virions. C, Virus with no added IgM. The abscissa in (a) shows the reciprocal of the IgM dilution.

antigenic sites. Maximum aggregation occurred at a dilution of 1/128. Aggregation was no longer detectable at a dilution of 1/4096. The last dilution (1/2048) which gave statistically significant aggregation ($P < 0.1$) was taken as the endpoint of aggregation. The mean number of virions per aggregate in the control was $1 \cdot 58 \pm 0.70$ ($n = 467$). The mean number of virions aggregated with 1/256 antibody was $1 \cdot 72 \pm 1.86$ ($n = 221$).

Comparison of Fig. 1(a) and 1(b) shows that IgM aggregated virions maximally between dilutions of 1/32 and 1/256 but did not neutralize the virus intrinsically. The absence of any loss of infectivity was evidently due to the efficacy of disaggregation during the preparation of dilutions for virus titration; without this, infectivity would have been diminished due to reduction in the number of infectious units. Similar results were obtained from two further experiments.

Detection of IgM bound virus by gold labelling

Aggregation of virus by IgM indirectly shows the presence of antibody. Here goat anti-mouse IgM antibody was used to detect directly mouse IgM bound to FPV/R at limiting dilution. The anti-antibody was tagged with 10 nm diameter colloidal gold particles which could easily be seen under the EM. The grids were then negatively stained and examined by EM. The number of gold grains over individual virions was counted and the mean results were plotted (Fig. 2). Binding of gold-labelled antibody increased with increasing IgM concentration but as found before (Taylor et al., 1987), this relationship was not directly proportional (compare with Fig. 3). Nonetheless comparison of Fig. 2 with Fig. 1 clearly shows that IgM is bound to virus at dilutions where virus was not neutralized. In the experiment shown the mean number of grains in the control was $1 \cdot 38 \pm 1.71$ ($n = 340$). The mean number of grains at 1/800 dilution ($2.09 \pm 2.31$, $n = 171$) was significantly greater than the control ($P < 0.01$). The mean number of grains at 1/1600 ($1.58 \pm 1.99$, $n = 57$) was not significantly different from the control ($P > 10$), therefore the endpoint for detection of bound IgM was taken as 1/800.

Binding of $^{125}$I-labelled IgM to virions

Fig. 3 shows that the binding of $^{125}$I-labelled IgM to FPV/R increased exponentially over the dilution range 1/30 to 1/1 with a 10-fold increase in radioactivity. Dilutions of 1/100 to 1/1 neutralized from 0 to 85% respectively. It was calculated that less than seven molecules of IgM are bound per virion at the dilution causing maximum aggregation; neutralization was initiated with approximately eight molecules of IgM per virion and reached a maximum of about 148 molecules per virion (Table 1).

The effect of IgM on the attachment of virions to the cell

Previous experiments (Taylor & Dimmock, 1985b) showed that a high (probably saturating) concentration of rabbit IgM abolished attachment of $^{32}$P-labelled FPV/R to BHK-21 monolayers at $4^\circ$C. A single
The binding of $^{125}$I-labelled IgM to FPV/R. Dilutions of IgM were incubated with FPV/R (■) or influenza B/Lee virus (▲) and the amount bound was determined after separation from free antibody by centrifugation. The extent of neutralization is also shown (○). Note that the concentration of IgM is given on the same relative scale as in Fig. 1. The arrow indicates maximum aggregation (see Fig. 1). The number of IgM molecules found is calculated as follows. 50% neutralization occurs at 1/8 dilution of the IgM preparation. The amount of $^{125}$I-IgM found is read off the far left ordinate as 37 IgM molecules per virion. The relationship between $^{125}$I radioactivity and IgM molecules is given in Methods.

Table 1. The numbers of IgM molecules per virion causing aggregation or neutralization

<table>
<thead>
<tr>
<th>Number of IgM molecules per virion</th>
<th>Experiment A</th>
<th>Experiment B</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum aggregation</td>
<td>&lt;7*</td>
<td>ND†</td>
<td>&lt;7</td>
</tr>
<tr>
<td>Initiation of neutralization</td>
<td>9</td>
<td>&lt;7</td>
<td>&lt;8</td>
</tr>
<tr>
<td>50% neutralization</td>
<td>33</td>
<td>37</td>
<td>35</td>
</tr>
<tr>
<td>80% neutralization</td>
<td>110</td>
<td>186</td>
<td>148</td>
</tr>
</tbody>
</table>

* Based on the binding of $^{125}$I-IgM: A and B are separate experiments. B is also shown in Fig. 3. The calculation is explained in the legend to Fig. 3.
† ND, Not determined.

Table 2. Attachment of virus neutralized by IgM to CEF cell monolayers

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Attached virus*</th>
<th>Attachment (%) control</th>
<th>Multiplication (%) control†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV</td>
<td>127</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>FPV + 1/1 IgM</td>
<td>18</td>
<td>14.2</td>
<td>56</td>
</tr>
<tr>
<td>FPV + 1/5 IgM</td>
<td>24</td>
<td>18.9</td>
<td>56</td>
</tr>
<tr>
<td>FPV + 1/25 IgM</td>
<td>103</td>
<td>81.1</td>
<td>100</td>
</tr>
</tbody>
</table>

* Number of virions attached to 50 cells.
† Percentage multiplication is determined as (100 - percentage virus infectivity neutralized).

The binding of FPV/R to CEF cells was examined directly by EM of cell sections in order to compare attachment of virus with infectivity. FPV/R was incubated with three different dilutions of IgM and then inoculated onto CEF cells at 4 °C. The cells were then pre-fixed and treated with an anti-HA IgG to ensure that virions would be tagged with antibody. These were then incubated with gold-labelled anti-IgG and anti-IgM antibodies and processed for EM as described in Methods.

Virions were easily located on the cell surface by the gold grains and the number per cell was noted. The results (Table 2) show a rough correlation between the concentration of IgM, giving 99% neutralization, was used in these experiments.
inhibition of the attachment of virions and the neutralization of the virus. A close correlation would not be expected due to the inherent variation in the size of the cell sections which depends on the position of the cut through the cell.

**Measurements of the height of the IgM layer attached to virus**

Virus which was neutralized by 83% by an undiluted preparation of IgM was examined by EM. The antibody was visible on the surface of virions as an uneven layer and the mean depth of the antibody fringe on virions was $19 \pm 5.9$ nm ($n = 40$). Note that IgM has a maximum height of about 10 nm in its ‘staple’ or ‘crab’ conformation and a maximum planar diameter of about 30 nm (Feinstein et al., 1971). Fig. 4 shows two virions from a preparation neutralized by 70%, where IgM molecules can be seen in various orientations to the virion surface. At lower, non-neutralizing, concentrations IgM was not visible which suggested that there were few antibody molecules present and that they were in the lower profile crab conformation.

**Discussion**

The IgM molecule is a pentamer comparable to five IgG molecules joined to form a central disc with five pairs of legs each of which carries an antigen-binding site. IgM molecules may bind to the antigen surface in two conformations (Feinstein et al., 1971). The antibody may be attached by several binding sites so that the central disc is parallel to the antigen surface in the crab or staple conformation. These IgM molecules have a height of up to 10 nm (Fig. 5b). In the second conformation, the IgM molecules are vertical to the surface and bound only by one or two binding sites. These molecules have a diameter of about 30 nm when fully extended (Fig. 5a). Since our preparation of IgM coated the virions to a mean depth of approximately 19 nm, the majority of the antibody molecules must be in the upright conformation, though the IgM is not necessarily completely extended. The approximate maximum diameter of an IgM molecule when bound in the crab or staple conformation with all five sets of ‘legs’ attached to the antigen surface is 25 nm (Feinstein et al., 1986). The influenza virion approximates a sphere with a diameter of 120 nm (Taylor et al., 1987), which would have a surface area of about 45000 nm$^2$. Comparison of the respective areas shows that a theoretical maximum of about 72 antibody molecules in the crab conformation would fit onto the surface of the virion, though our results suggest that this number is approximate and may exceed the theoretical maximum (Taylor et al., 1987).

Any increase in the number of IgM molecules per virion above the theoretical saturating number of 72 crab conformational states must mean that an increasing proportion of them are attached in a more vertical position which permits closer packing. This was verified experimentally as we found that the number of IgM molecules causing 80% neutralization was about 148 per virion.

FPV has 1000 HA and 300 neuraminidase spikes which are 5-1 nm apart (Taylor et al., 1987). In Fig. 5(a) IgM and HA spikes are drawn to scale at a ratio of 172 IgM molecules per virion (one IgM to 7.5 spikes) which gives about 90% neutralization. For comparison a section of the surface of a virion with the maximum density of 72 IgM crabs per virion (66% neutralization; see above for calculations) is shown in Fig. 5(b) and again for comparison with 72 IgG molecules per virion (Fig. 5c). In the latter case the surface of the virus is
sparsely covered yet virus is neutralized by about 63% (Taylor et al., 1987). How infectivity is lost has already been covered in the Introduction.

EM examination of virions saturated with IgM showed that the virus has an irregular heavy fringe of antibody with a mean depth of 19 nm, so that the majority of molecules must be in the upright position, as in Fig. 5(a), although the IgM is not necessarily completely extended. Since even heavily coated virus preparations are neutralized to only around 87% it is likely that areas on some virions are incompletely coated with IgM or complexed with IgM crabs, thus allowing cell receptor units to penetrate the IgM layer and attach the virus.

While binding of IgM to virus increased exponentially, neutralization increased in an approximately linear fashion implying that additional IgM molecules were binding to virus which had already been neutralized. Under the conditions used we did not observe any decrease in the amount of IgM-binding at high concentrations of antibody, presumably because the IgM concentration was not great enough. The typical neutralization curve (Fig. 3) was usually biphasic with an inflection at around 50% neutralization, when about 35 molecules of IgM are bound per virion. It is possible that this reflects the change from the crab to the planar conformation of the antibody.

IgG is postulated to neutralize by binding to 'neutralization-relevant' spikes (Taylor et al., 1987). As at 63% neutralization there was not one but 70 IgG molecules per virion (Fig. 5c), 70 to 140 spikes were involved depending on whether the antibody was attached to one spike or two. The same ratio of IgM molecules to virus gives approximately 66% neutralization yet each IgM molecule would bind to one to 10 spikes and involve 70 to 700 spikes per virion, including presumably neutralization-relevant spikes. Saturated virus is incompletely neutralized, although antibody must be bound to hundreds of spikes. This further indicates that IgG and IgM act by different mechanisms.

The data presented here and those of Taylor & Dimmock (1985b) and Outlaw & Dimmock (1990) suggest that neutralization of influenza virus by IgM is dependent on steric hindrance, but in vivo, other mechanisms such as aggregation of virus may help to reduce infectivity. We have shown that maximum aggregation requires less than seven molecules of IgM per virion, considerably less than is required for neutralization of individual virions. Aggregation occurs only at low IgM concentrations when antigenic sites are available on adjacent virus particles and requires that the legs of the IgM crab flex in opposite directions (Feinstein et al., 1971) to make the necessary cross-link. However virus–IgM aggregates appear to be unstable as compari-

References


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TAYLOR, H. P. & DIMMOCK, N. J. (1985a). Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. *Journal of Experimental Medicine* 161, 198-209.


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