Mechanisms of Neutralization of Influenza Virus by IgM

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SUMMARY

IgM which neutralized influenza virus infectivity by over 99% prevented the attachment of only half the virus population to BHK cells at 37 °C. However, the half of the population that attached to cells was not internalized. Loss of infectivity brought about by IgM is thus totally different from that caused by neutralizing IgG which does not inhibit attachment or penetration.

Protection against influenza infection is largely due to the presence of neutralizing antibody directed to the haemagglutinin (HA) of influenza virus (Hobson et al., 1972; Virelizier, 1975; for review see Ennis, 1982). The immunoglobulins IgG, IgA, secretory (s) IgA, and IgM all have neutralizing activity (Ogra et al., 1975) and each probably contributes to resistance to influenza (Waldman et al., 1967; Shvartsman & Zykov, 1976; Couch & Kasel, 1983). Secretory IgA and IgM are thought to be of particular importance in immunity to influenza, as both are enriched in secretions relative to IgG (Tomasi et al., 1965; Brandtzaeg et al., 1970). However, nearly all information on the way in which antibody neutralizes the infectivity of influenza or other animal viruses is based on the study of IgG (Dimmock, 1984) and only recently has the work been extended to IgA (Taylor & Dimmock, 1985).

In order to neutralize influenza virus, it is evident that antibody (IgG) has to bind not just to the HA (Seto & Rott, 1966; Webster & Laver, 1967) but to certain specific regions of it (Webster & Laver, 1980; Gerhard et al., 1981; Wiley et al., 1981; Caton et al., 1982) as there are other antibodies which bind to the HA but do not neutralize (Breschkin et al., 1981; H. P. Taylor & N. J. Dimmock, unpublished data). The latter are presumably directed to different regions of the HA molecule. Neutralization of influenza virus by IgG was not mediated through inhibition of attachment, penetration, uncoating and transport of the viral genome to the nucleus, as these were unaffected by neutralization to over 99-99%, by monoclonal or polyclonal IgG (Possee et al., 1982; Dimmock et al., 1984). These findings were unaffected over a range of temperatures from 4 °C to 37 °C (H. P. Taylor & N. J. Dimmock, unpublished data). Neutralization, it was thought, must affect some stage subsequent to the nuclear accumulation of the virus genome. Using the same system we have found more recently that about half the influenza virus neutralized by slgA is prevented from binding to BHK cells but, in contrast to the situation with IgG, neutralized virus attached to cells remains on the outside of the cell and is not internalized (Taylor & Dimmock, 1985). However, monomers of IgA prepared by differential reduction of slgA neutralized virus in the same manner as IgG. Presumably the unique physical and biological properties of the immunoglobulins are responsible for these differences. In this report we extend the comparison to IgM and describe how the purified antibody neutralized influenza virus by reducing attachment of the virus population to BHK cells and preventing the remainder from being internalized. In these regards neutralization by IgM and IgG (Dimmock et al., 1984) are totally different.

The avian influenza virus A/FPV/Rostock/34 (FPV/R; H7N1) was radiolabelled with $^{32}$P (Amersham) by infecting chick embryo fibroblast cell monolayers in roller bottles in the presence of the isotope (Possee et al., 1982). $^{32}$P-labelled virus was precipitated from tissue culture fluid as described by Dimmock et al. (1977) except that no unlabelled carrier virus was added. BHK-21 cell monolayers were cultivated in roller bottles in Glasgow minimum essential medium (Gibco-BRL) supplemented with 100 units penicillin, 100 μg streptomycin sulphate,
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0.295 mg tryptose phosphate per ml and 5% (v/v) newborn calf serum (Gibco-BRL). When confluent, cells were removed with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate-buffered saline (PBS) and re-seeded in roller bottles in 51 mm diameter Nunclon plastic Petri dishes (Gibco-BRL) at a concentration of 6 × 10⁶ cells/dish. The virus used to inoculate rabbits was the reassortant (V31; H7N2) obtained by co-inoculating CEF cells with a mixture of A/FPV/Rostock/34 (H7N1) and A/X49 (H3N2) (Rott et al., 1976). In this way antibody prepared was specific for the HA of A/FPV. Rabbits (2 kg half-lops: Hylyne Rabbits Ltd, Northwich, U.K.) were injected with 10⁵ haemagglutination units (HAU) of purified V31 intravenously and serum was obtained daily between days 2 and 9 post-inoculation. IgM was purified using an affinity column constructed by coupling goat anti-rabbit IgM (Fc) (Nordic Immunological Laboratories Ltd., Maidenhead, U.K.) to cyanogen bromide-activated Sepharose (Sigma). Serum (4 ml) was passed through the column with 0.01 M-Tris-HCl, 0.15 M-sodium chloride, pH 8. The column was extensively washed with the same buffer and then IgM was eluted with sodium acetate buffer solution (pH 3). Eluted samples were adjusted to pH 7 with NaOH, dialysed overnight against 1 mM-Tris-HCl, 0.05 M-sodium chloride, pH 7.4, then lyophilized. IgM was characterized biochemically by SDS-polyacrylamide gel electrophoresis and immunologically by the Ouchterlony test. Electrophoresis through 5 to 30% (w/v) acrylamide under non-reducing conditions gave a single strong band of about 900 000 mol. wt. at the expected position of the IgM pentamer. The preparation reacted with anti-μ serum in double diffusion in agar but not with anti-γ (data not shown). Serum obtained from bleeds at days 6 and 7 produced maximum yields of IgM as monitored by haemagglutination inhibition (HI) titrations with chicken erythrocytes. Neutralization by IgM was measured by the reduction in infectivity of FPV/R as determined by plaque assay on CEF cell monolayers under 0.9% agar in 199 Medium containing 5% newborn calf serum.

The attachment of non-neutralized and neutralized ³²P-labelled FPV/R to BHK-21 cells was measured as described previously (Dimmock et al., 1984). Virus was first incubated for 1 h at 25 °C with neutralizing (n) IgM or an equivalent mass of IgM from pre-immunization serum (pIgM). One-hundred μl volumes of virus–IgM mixtures were inoculated onto monolayers of BHK-21 cells in plastic Petri dishes. Initial experiments used monolayers held on ice (giving a temperature of 4 °C), as this temperature allows the genome of influenza virus to enter nuclei but prevents any virus-directed macromolecular synthesis from taking place (Stephenson & Dimmock, 1975). Subsequent experiments used cells held at 25 °C or 37 °C in water-baths. At the required intervals, monolayers were washed three times with cold PBS and removed by scraping. Radioactivity associated with the cells and washes was determined by binding to Whatman DE81 filter discs: extracts were spotted onto filters which were then washed three times with sodium dihydrogen orthophosphate and finally with water. This enables measurement of ³²P-labelled RNA; lipids and proteins do not bind to DE81 filters. At 4°C, nIgM prevented the attachment of neutralized virus (Fig. 1) whereas over 25% of infectious virus which had been incubated with pIgM attached to BHK-21 cells by 120 min. At 25°C and 37°C similar amounts of non-neutralized virus attached as at 4°C but attachment of neutralized virus rose at 25°C to 38% and at 37°C to 47% of their respective control values after 120 min (Fig. 2). To investigate whether virus neutralized with IgM was able to undergo any of the subsequent stages of infection as it does when neutralized with IgG, we determined the levels of virion RNA which accumulated in nuclei. Nuclear and cytoplasmic fractions were obtained by incubating monolayers with 0.5% NP40 (BDH) in 0.25 M-sucrose, 0.01 M-Tris–HCl and 1 mM-MgCl₂ (pH 7.4) for 5 min on ice. After repeating this procedure it was found that the majority of cytoplasm had been removed, leaving nuclei attached to the Petri dish. These were scraped off and further purified as described by Possee et al. (1982) and Dimmock et al. (1984). Recovery of nuclei was 94% and contamination of nuclei by cytoplasm was <5%. Radioactivity was collected as above using Whatman DE81 filters. Fig. 3 shows that even though a substantial proportion of virus neutralized with IgM attached to cells at 25°C and 37°C, virion RNA did not travel to the nucleus. However, this procedure does not allow us to discriminate between virus attached to the outside of the cell and virus which has been internalized in the cytoplasm. To remove virus putatively attached to the outside, cells were digested with proteinase K. ³²P-
Fig. 1. Kinetics of attachment of non-neutralized $^{32}$P-labelled FPV/R and virus neutralized with IgM to BHK-21 cells at 4°C. Virus was first incubated for 1 h at 25°C with neutralizing IgM (●) or pre-immune IgM (○). Infectivity was neutralized (by 99%) only by the former. Virus–IgM mixtures were chilled and 100 μl inoculated onto the monolayers of BHK-21 cells in Petri dishes (6 × 10⁶ cells/dish) held on ice. Each 100 μl initially contained 1.5 × 10⁷ p.f.u., 6.3 × 10² HAU and 1.34 × 10⁵ c.p.m. At each time interval, monolayers were washed thoroughly with cold PBS before scraping the cells from the dish. $^{32}$P-labelled virion RNA was collected on DE81 filters. The ordinate shows the amount of radioactivity attached to cells as a percentage of that inoculated.

Fig. 2. Kinetics of attachment of non-neutralized and neutralized $^{32}$P-labelled FPV/R to BHK-21 cell monolayers at various temperatures. Virus was first incubated for 1 h at 25°C with neutralizing IgM (solid symbols) or pre-immune IgM (open symbols). The neutralizing IgM reduced infectivity by 99%. Each 100 μl of inoculum initially contained 1.5 × 10⁷ p.f.u., 6.31 × 10² HAU and 1.34 × 10⁵ c.p.m. Neutralizing IgM at 4°C (●), 25°C (▲), 37°C (■); pre-immune IgM at 4°C (○), 25°C (▲) and at 37°C (□).

Labelled virus–IgM mixtures were incubated with monolayers for 2 h at 25 or 37°C, and cells were then rinsed three times with cold PBS and incubated at 4°C with 500 μl proteinase K (2 μg/ml; BDH, sp. act. 20 Anson units/g) for 60 min. The enzyme solution was removed and pooled with two subsequent washes of 250 μl PBS. Fig. 4 shows that 80% or more virion RNA was released from those cells which had been inoculated with virus neutralized by nIgM compared with <20% released from cells receiving non-neutralized virus. This clearly demonstrates that the majority of attached neutralized virus is remaining on the outer surface of the cell.

In conclusion, this study shows that despite its large size, IgM did not stop nearly half of the neutralized virus from attaching to cells at physiological temperatures. However, in contrast to virus neutralized with IgG or IgA (Possee et al., 1982; Dimmock et al., 1984; Taylor & Dimmock, 1985), it was not internalized. Thus IgM resembles sIgA (Taylor & Dimmock, 1985) as it appears to interfere, in some unknown way, with the endocytic event responsible for internalization of virus (Matlin et al., 1981).

Attachment of virus neutralized by IgM and sIgA but not by IgG or IgA (Dimmock et al., 1984; Taylor & Dimmock, 1985) was greatly temperature-dependent and it may be that at 4°C, the pentameric IgM molecule interferes with attachment by virtue of its considerably larger...
Fig. 3. Kinetics of migration of $^{32}$P-labelled virion RNA from cytoplasm to nucleus in cells inoculated with virus neutralized by IgM. Virus was neutralized and inoculated at 4°C, 25°C and 37°C as described in Fig. 2. Neutralizing IgM at 4°C (●), 25°C (▲), 37°C (■); pre-immune IgM at 4°C (○), 25°C (△) and 37°C (□).

Fig. 4. Release of $^{32}$P-labelled virus neutralized by IgM from BHK-21 cells by incubation with protease K. Cells were inoculated with virus treated with pre-immune (p) IgM or neutralizing (n) IgM as described in Fig. 2. One-hundred μl of virus–antibody mixtures were incubated with BHK-21 cells at the temperatures shown (°C) for 120 min. Cells were then washed twice with cold PBS and incubated with 500 μl protease K (2 μg/ml) in PBS or 500 μl PBS for 60 min at 4°C. The percentage $^{32}$P released by protease K or by PBS was calculated relative to the total $^{32}$P associated with cells before digestion was carried out.

molecular structure. At higher temperatures, increased thermal agitation could be responsible for bringing receptor and target molecules into the correct juxtaposition.

Clearly the mechanism of neutralization of influenza virus depends upon the class of antibody involved and this may well apply to neutralization of viruses belonging to other families. There is no evidence from which to generalize and each virus must be investigated to determine the nature of the virus–antibody–cell interaction. One implication of our data which merits further study is the possibility that cells to which influenza virus neutralized with IgM has attached, may be open to attack by complement or other elements of the immune system and that these may contribute to the pathology of infection.

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REFERENCES

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