Mechanisms of neutralization of a nairovirus (Dugbe virus) by polyclonal IgG and IgM

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Dugbe virus is a member of the nairovirus genus of the *Bunyaviridae*. Purified polyclonal anti-Dugbe virus IgG, which neutralized >99.5% of virus, reduced attachment of virus to BSC-1 cell monolayers by only 36%. A 100-fold lower concentration neutralized virus by 88%, and had no effect upon attachment. Neutralizing IgG did not affect the ability of Dugbe virus to be internalized by or to fuse with BSC-1 cells. This suggests that IgG neutralization occurs largely at a stage subsequent to primary uncoating. Purified polyclonal anti-Dugbe virus IgM neutralized infectivity and had no effect on the attachment of virus to cells, but inhibited internalization of virus by about 50%. Thus IgM neutralizes partly by interfering with entry of virus and partly by a post-entry event. Neutralization by intermediate concentrations of IgM was enhanced 20-fold in the presence of complement. At high concentrations of IgM, complement-dependent neutralization declined. This is probably due to IgM binding in a planar rather than crab conformation, which does not expose the complement binding sites. Aggregation occurred only at relatively low concentrations of immunoglobulin. Electron microscopy and reactivation of infectivity by vortexing suggested that aggregation makes only a minor contribution to neutralization by IgG or IgM.

Introduction

Dugbe virus is a tick-borne arbovirus causing febrile illness in vertebrates in Africa. It belongs to the nairovirus genus of the *Bunyaviridae* (see review by Elliott, 1990), members of which contain three segments of negative sense ssRNA (Clerx *et al.*, 1981). Dugbe virus induces six polypeptides (Cash, 1985), two of which are non-structural and two external glycoproteins. Little is known of the structure of the latter, but one or both are presumed to carry neutralizing epitopes, as described for La Crosse virus, a member of the bunyavirus genus (Kingsford & Hill, 1983; Kingsford *et al.*, 1983; Kingsford & Ishizawa, 1984). Kingsford *et al.* (1991) have studied the mechanism of IgG neutralization of La Crosse virus.

Neutralization by immunoglobulins has been extensively investigated, but the principles by which inactivation of infectivity is brought about have been difficult to define (Dimmock, 1984, 1987). However, it is clear that there are several different mechanisms of neutralization, and it is probably true that every stage in the early interaction of virus and cell is a potential target for neutralization, the target being decided by a particular combination of virus, viral epitope, isotype and concentration of immunoglobulin, and the cell type. In fact, work on La Crosse virus was among the first to show unequivocally the importance of the cell as a determinant of neutralization (Grady & Kinch, 1985). Most is known about IgG neutralization which is usually, but not always, mediated by a post-attachment mechanism (N. J. Dimmock, unpublished data); there is little information in general about how IgM and IgA mediate neutralization. Information about mechanistic aspects of the neutralization of enveloped viruses has come mainly from the study of type A influenza virus (Outlaw & Dimmock, 1991; Armstrong & Dimmock, 1992) and human immunodeficiency virus type 1 (HIV-1) (McKeating & Willey, 1989; Putney & McKeating, 1990; Nara *et al.*, 1991). Here we have used an enveloped virus from another family, the *Bunyaviridae*, and studied its neutralization by polyclonal IgG and IgM.

Methods

*Virus growth and purification.* Dugbe virus, strain KT281/75, was obtained from Dr P. Cash, Department of Bacteriology, University of Aberdeen Medical School, Aberdeen, U.K. Plaque-purified virus was grown in confluent monolayers of BSC-1 cells in the Glasgow modification of Eagle's medium (GMEM) containing 5% newborn calf serum. Virus growth and purification conditions were carefully optimized for yield, purity and infectivity using PAGE, electron microscopy and calculation of particle:p.f.u. ratios (data not shown).
This was necessary as virus was relatively labile, losing 50% infectivity after 2 h at 37 °C, 9 h at 25 °C and > 30 h at 4 °C. The procedure finally adopted was as follows. Cells were inoculated with virus at an m.o.i. of 0.01 for 1 h at 33 °C, after which the inoculum was removed and replaced with serum-free medium. Tissue culture fluid (TCF) was harvested 28 h post-infection, clarified at 1500 g for 20 min at 4 °C, and concentrated using an Omegacell with a 10^4 M, cut-off (Filtron). Concentrated TCF was loaded onto step gradients containing equal amounts of 50% and 30% urografin (Seringer) in TNE buffer (10 mM-Tris–HCl, 0.1 M-NaCl, 1 mM-EDTA pH 7.5). Gradients were centrifuged at 110000 g for 17 h at 4 °C, and a visible band of virus at the interface between the urografin layers was collected. This was dialysed against PBS at pH 7, concentrated using Centrinoc Microconcentrators (with a 3 x 10^4 M, cut-off; Amicon) and stored at -70 °C.

Virus titration and neutralization assays. Infectivity of virus was determined by plaque assay on 35 mm diameter BSC-1 cell monolayers under 0.9% agar in GMEM containing 0.04% DEAE-dextran and 2.5% foetal calf serum. After 5 days incubation at 37 °C, monolayers were fixed with 5% glutaraldehyde and stained with 1% crystal violet. The neutralizing titre of antibody was assessed by plaque reduction, with the required amount of virus being incubated with purified immunoglobulin at 25 °C for 3 to 4 h. Dilutions were then made for titration. The long incubation time was needed to obtain maximum neutralization.

Preparation of 32P-labelled virus in vitro. BSC-1 cell monolayers were incubated in phosphate-free GMEM for 24 h and then infected with Dugbe virus. Inoculum was removed and virus grown in the presence of inorganic 32P (2.5 mCi in 30 ml medium; Amersham) and harvested 28 h later. Radiolabelled virus was purified by centrifugation through urografin. Virus was stored at -70 °C for up to 2 weeks and lost no more than 50% infectivity over this period. A typical preparation of purified virus contained 8 x 10^6 p.f.u./ml and 4.5 x 10^6 c.p.m./ml.

Biotinylation of virus in vitro. The surface proteins of purified Dugbe virus were labelled with biotin by addition of biotinylation reagent (4 μl/mg protein, Amersham) using the protocol provided by the manufacturer. Biotinylated virus was stored at -70 °C and retained on average 64% of its initial infectivity.

Production and purification of anti-Dugbe virus IgM and IgG. Rabbits were inoculated with urografin-purified virus: at day 0 with 1 x 10^6 p.f.u. injected intravenously (i.v.), at day 15 with 1 x 10^7 p.f.u. in Freund’s complete adjutant injected subcutaneously, and on day 58 with 1 x 10^8 p.f.u. injected i.v. IgM was purified by gel filtration on Sephacryl S300 (Pharmacia) from serum taken 7 days after the primary inoculation. Purified IgM was concentrated using Centrinoc 30 microconcentrators and stored at 4 °C. IgG was purified by affinity chromatography on Protein-A Sepharose (Sigma) from serum taken on day 65. IgG was concentrated using Centrinoc 30 micro-concentrators and stored in aliquots at -20 °C. The purity of polyclonal IgG and IgM was checked by PAGE. The isotype was verified using a dip-stick assay (Amersham). Mouse monoclonal IgG2b specific for Dugbe virus G1 glycoprotein was purified by the same method from H28.17 ascitic fluid kindly provided by Dr E. A. Gould (NERC Institute of Virology and Environmental Microbiology, Oxford, U.K.); H28.17 is neutralizing and protects mice on passive transfer (E. A. Gould, unpublished data). Protein concentrations were determined using a protein assay kit (Bio-Rad); purified polyclonal IgM was adjusted to 2 mg/ml, polyclonal IgG to 16 mg/ml and monoclonal IgG to 0.7 mg/ml.

Assay for attachment of virus to cells. 32P-labelled virus or neutralized 32P-labelled virus (200 μl) was inoculated onto 35 mm diameter BSC-1 cell monolayers and incubated at room temperature. Inoculum was removed at intervals and monolayers were washed eight times with PBS. Cells were removed by trypsinization and radioactivity in the cell suspension was determined in a liquid scintillation counter. About 14% of the free virus bound to cells in 2 h.

Assay to determine internalization of virus. Biotinylated virus or neutralized biotinylated virus (40 μl) was inoculated onto BSC-1 cell monolayers in flat-bottomed 96-well tissue culture plates and incubated at room temperature. At intervals the inoculum was removed and cells were washed three times with PBS. Monolayers were then fixed and permeabilized for 1 h at room temperature in 1:1 acetone : methanol to permit the assay of total cell-associated biotinylated virus, or with 1% glutaraldehyde for assay for biotinylated virus on the exterior surface of the cell. Plates were then incubated with 1% BSA in PBS and biotin was measured by ELISA using streptavidin linked to alkaline phosphatase or horseradish peroxidase. The extent of internalization of virus was calculated as the difference in absorbance (A) between total cell-associated biotinylated virus and external biotinylated virus. Five replica wells were used and the results were averaged for each time interval.

Virus-induced cell fusion at low pH. The method of Yoshimura et al. (1982) was used. Purified virus was incubated with purified immunoglobulin or PBS for 1 h at room temperature. Virus (20 μl containing 100 p.f.u., or pre-neutralization p.f.u./cell) was adsorbed to an equal volume of a suspension of 5 x 10^6 BSC-1 cells/ml for 1 h at 4 °C. Fusion was induced by resuspending the cells in 10 mm-MES buffer at pH 6.0 (the optimum pH for Dugbe virus fusion; unpublished data), incubating for 30 min at 37 °C with shaking, cells were then counted using a haemocytometer. A reduction in cell number indicated that cell fusion had occurred. A fusion index was calculated as follows using the appropriate controls.

Fusion index = [(total cell number/total cell number after incubation with virus) – 1]

Complement-dependent neutralization. Purified virus (2 x 10^7 p.f.u.) was pretreated with a range of concentrations of IgG or IgM and incubated with 4 units of complement (Cedarlane Laboratories) for 1 h at 37 °C, and the extent of neutralization was determined by plaque assay. The presence of free complement was determined by titration using a conventional complement fixation assay with sensitized red blood cells.

Assay of virus aggregation. Purified virus (5 x 10^8 p.f.u./ml) was incubated with dilutions of antibody for 1 h at room temperature. The samples were divided and sample 1 was vortexed for 3 s, diluted and plated out on BSC-1 cells for plaque assay; sample 2 was diluted without vortexing and plaque assayed; sample 3 was fixed for electron microscopy in 2% paraformaldehyde overnight at 4 °C. The fixed cells were mixed with 3% sodium silicotungstate pH 7.0, dropped onto Formvar grids, drained after 5 min and examined under a JEOL 100S transmission electron microscope. Single virions and the number of virions in each aggregate were counted and averaged. Aggregates were easily disrupted by vortexing (data not shown).

Results

IgG neutralization of Dugbe virus has little if any effect on attachment to or internalization by BSC-1 cells

A diluted preparation of purified rabbit polyclonal IgG neutralized virus by 88% and did not significantly inhibit the rate of attachment of virus. A 100-fold greater concentration neutralized virus by >99% and reduced
IgG and IgM neutralization of Dugbe virus

attachment by only 36% (Fig. 1). The same high concentration of IgG did not inhibit the rate of internalization (Fig. 2). A similar conclusion was reached using purified mouse monoclonal IgG which neutralized virus by >90% (data not shown). Therefore IgG neutralization occurs largely by a post-internalization mechanism.

IgM neutralization of Dugbe virus does not inhibit attachment to, but partially inhibits internalization by BSC-1 cells

There was no significant inhibition of attachment in terms of rate or final amount over a 100-fold range of neutralizing polyclonal IgM concentration (Fig. 3). However, neutralizing IgM reduced the rate of internalization and inhibited its extent by around 50% after 60 min incubation (Fig. 4). Although this will contribute to neutralization, it is not sufficient to account for the extent observed, thus some neutralization also occurs by a post-internalization mechanism.

Neither IgG nor IgM inhibits low pH-mediated virus-induced fusion of BSC-1 cells

Positive controls (virus-coated cells incubated at pH 6.0) had a fusion index of 0-41. Negative controls (at pH 7.0) showed no fusion and therefore had a fusion index of 0. Fig. 5(a, b) shows that a range of concentrations of neutralizing IgG or IgM had no effect on the ability of the virus to cause cell fusion (Fig. 5a, b).

Complement-mediated IgG and IgM neutralization of Dugbe virus

Fig. 6(a) shows that complement increased the neutralizing activity of IgG by about threefold. Back-titration of free complement by the addition of sensitized red blood cells showed that removal of complement correlated with a decrease in neutralization; the plateau of complement removal coincided with over 80% neutralization. When complement-independent neutralization reached 90%, the addition of complement did not result in any further loss of infectivity (Fig. 6b).

Complement increased the neutralizing activity of
Fig. 3. Kinetics of attachment to BSC-1 cells of Dugbe virus neutralized by purified polyclonal IgM. Virus-antibody mixtures originally containing 1 x 10^6 p.f.u./ml ^3^P-labelled virus were incubated for 4 h at 25 °C. Neutralized or non-neutralized virus was inoculated onto monolayers and then incubated. At each time point monolayers were washed thoroughly with cold PBS, cells were removed by trypsinization and total radioactivity in the cell suspension was counted. Purified IgM from an irrelevant immune serum was used as control. O, virus + non-specific IgM (1:10), 0% neutralization; •, virus + IgM (1:10), 90-7% neutralization; □, virus + IgM (1:100), 99% neutralization; ■, virus + IgM (1:1000), 93% neutralization.

Fig. 4. Kinetics of internalization by BSC-1 cells of Dugbe virus neutralized by purified polyclonal IgM. Virus-antibody mixtures originally containing 2 x 10^6 p.f.u./ml biotinylated virus were incubated for 3.5 h at 25 °C. Neutralized or non-neutralized virus was inoculated onto cell monolayers and then incubated. At each time point monolayers were washed and fixed as described in Methods, and the amount of cell-associated biotinylated virus was determined by ELISA using an alkaline phosphatase detection system. Internalization of virus was calculated as described in legend to Fig. 2. O, virus + irrelevant IgM (1:10), 0% neutralization; •, virus + IgM (1:10), >90% neutralization.

IGM by up to 20-fold (Fig. 7a). Back-titration of free complement showed that fixation of complement by IgM–Dugbe virus complexes declined when the highest concentrations of IgM were used (Fig. 7b). The residual non-neutralized fraction was at a minimum when virus was neutralized in the presence of complement.

Aggregation and neutralization: IgG

Vortexing of IgG-neutralized virus reduced the neutralizing ability of dilutions of IgG by about fourfold over a range of dilutions from 1:16 to 1:256 (Fig. 8a). However, at high concentrations of IgG (<1:4) infectivity could not be reactivated. Quantitative electron microscopy showed that IgG aggregated virus over the same range of dilutions, giving a mean maximum aggregate size of 1-4 virus particles above background at a 1:64 dilution (Fig. 8b). This is sufficient to account for the higher level of neutralization observed with non-vortexed samples (Fig. 8a). This view is supported by the reactivation of infectivity which occurred when the virus–IgG mixture was vortexed. As expected, aggregation was reduced at high IgG concentrations (1:8); the virus is coated so heavily with antibody that there are no free epitopes to
IgG and IgM neutralization of Dugbe virus

IgG and IgM neutralization of Dugbe virus by IgG. Virus and dilutions of purified IgG were incubated for 1 h at room temperature, and then incubated with or without 4 units of complement for 45 min at room temperature. Mixtures were then vortexed and the extent of neutralization was determined by plaque assay (a). Neutralization by IgG + complement (▲); neutralization by IgG (○). The presence of free complement at the end of this incubation was determined by lysis of sensitized red blood cells (b). Thus 100% complement fixation (CF) indicates that the virus–antibody complex had reacted with all the complement added.

Fig. 6. Complement-enhanced neutralization of Dugbe virus by IgG.

IgG and IgM neutralization of Dugbe virus by IgM. Virus and dilutions of purified IgM were incubated for 1 h at room temperature, and then incubated with or without 4 units of complement for 45 min at room temperature. Mixtures were then vortexed and the extent of neutralization was determined by plaque assay (a). Neutralization by IgM + complement (▲); neutralization by IgM (○). The presence of free complement at the end of this incubation was determined by lysis of sensitized red blood cells (b), see Fig. 6.

Fig. 7. Complement-enhanced neutralization of Dugbe virus by IgM.

Aggregation and neutralization: IgG

Vortexing of IgM-neutralized virus had only a minor effect on the extent of neutralization and was most marked between dilutions of 1:8 and 1:32 at which up to 25% of the neutralization was reversed. Vortexing had no reactivating effect above a level of 50% neutralization (Fig. 9a). Electron microscopy showed a low degree of aggregation, with a maximum of 24 virions per aggregate at a 1:32 dilution of IgM, whereas the control without antibody averaged 1-4 virions per aggregate.

Fig. 8. Aggregation of Dugbe virus by IgG. (a) The effect of disaggregation by vortexing on neutralization. The difference in titre is also plotted. (b) An electron microscope study to determine the extent of aggregation (average number of virus particles/aggregate) as a function of IgG concentration compared with a non-neutralized sample (---). Neutralization by IgG after vortexing (○); neutralization by IgG before vortexing (▲); neutralization (%) before vortexing – neutralization (%) after vortexing (〇): right-hand axis.

Discussion

Neutralizing IgG had very little effect on the ability of Dugbe virus to attach to BSC-1 cells, and no effect on its ability to be internalized or to fuse with them. The fusion
of cells by externally attached virus at an artificially lowered pH is used here as a model of intra-endosomal fusion of viral and vesicular membranes. The fact that fusion occurs optimally at pH 6 (data not shown) suggests that the virus is uncoated at low pH in the endosomal compartment rather than at neutral pH at the plasma membrane (Marsh & Helenius, 1989). Therefore, it is likely that little IgG in this polyclonal preparation is directed against the virus attachment site or to any part of the virus concerned with fusion. IgG neutralization is thus mediated by a post-internalization, and possibly by a post-uncoating, mechanism. There was little aggregation of Dugbe virus and that was over only a narrow range of low IgG concentrations, recalling the situation with influenza virus (Outlaw et al., 1990). For the latter virus we know that antibody is not made to the virus attachment site (Wilson et al., 1981) and that virus is neutralized by a post-uncoating mechanism (Rigg et al., 1989; Outlaw & Dimmock, 1991). There are also similarities with the IgG neutralization of La Crosse virus, a member of the bunyavirus genus of the Bunyaviridae. The G1 protein of La Crosse virus has eight antigenic sites (Kingsford et al., 1983). There are neutralizing monoclonal antibodies against seven of these sites, as determined by assay on BHK cells, but only those to regions A and G neutralize efficiently; those against region A inhibit attachment of the virus, but those against region G do not interfere with attachment and are thought to affect an event within the cell (Kingsford et al., 1991). The anti-Dugbe virus IgG resembles the latter. However, this resemblance may be coincidental as nairo- and bunyaviruses have different surface subunit structures, for instance the diameter of La Crosse spikes being around fivefold greater than those of Dugbe (Martin et al., 1985).

Neutralizing IgM did not affect attachment of Dugbe virus to BSC-1 cells or its ability to cause cell fusion. It did cause a 50% drop in internalization, but this was insufficient to account for the degree of neutralization observed. Thus IgM neutralization is caused partly by inhibition of internalization and partly by a post-uncoating event (with the reservation already discussed above). Again there was little aggregation and this was restricted to a particular virus: antibody ratio, as noted earlier for IgM neutralization of poliovirus (Thomas et al., 1986). This situation is not dissimilar to the IgM neutralization of influenza virus, except with this virus there is some inhibition of attachment, although not enough to account for neutralization (Taylor & Dimmock, 1985; Outlaw & Dimmock, 1990; Armstrong et al., 1990). The limited data available from other systems confirm that IgM neutralization generally results from partial inhibition of attachment and/or internalization (poliovirus, Mandel, 1967; murine cytomegalovirus, Farrell & Shellam, 1990). Vaccinia virus partially neutralized by IgM attaches to and enters cells but is not uncoated (Rodriguez et al., 1985). IgM directed to the membrane-bound portion of the HIV-1 glycoprotein (gp41) is also neutralizing, but its mechanism of action has not been investigated (Dalgleish et al., 1988).

Dugbe virus is about 100 to 120 nm in diameter (S. J. Armstrong, data not shown) and IgM about 30 nm in diameter (Feinstein et al., 1971, 1986). What are the circumstances which prevent high concentrations of such a large molecule from interfering with the interaction of Dugbe virus and BSC-1 cell receptors? We can surmise that the charge imposed by IgM is not unfavourable to interaction with the cell, that IgM is not directed to the virus attachment site, that cell receptors are long enough to interdigitate with IgM (Dimmock et al., 1987) and that the part(s) of the envelope concerned with fusion is unimpeded. IgM in its ‘crab’ or ‘staple’ conformation extends only about 10 nm above the surface of the virus (Feinstein et al., 1971, 1986), and many cell surface molecules, potential virus receptors, are longer than this (Springer, 1990). IgG is of a similar height and extends between 8 and 16 nm above the surface of a virion depending on the angle of flexion of the Fc region (Silverton et al., 1977).

Fig. 9. Aggregation of Dugbe virus by IgM. (a) The effect of disaggregation by vortexing on neutralization. The difference in titre is also plotted. (b) An electron microscope study to determine the extent of neutralization (average number of virus particles/aggregate) as a function of IgM concentration compared with a non-neutralized sample (---). Neutralization by IgM after vortexing (●); neutralization by IgM before vortexing (▲); neutralization (%) before vortexing – neutralization (%) after vortexing (○): right-hand axis.
Although our polyclonal IgG and IgM preparations both neutralized independently of complement, neutralization was enhanced three- to 20-fold, respectively, when complement was present. The small increase in IgG neutralization is not due to the failure of anti-Dugbe virus IgG to bind complement as all the added complement was used up. Perhaps C1q, the first component of the complement cascade, is bound only monovalently by Fc moieties which are too far apart on the surface of the virion to permit the divalent binding, which is the minimum needed for activation. The much greater (20-fold) enhancement by complement of IgM-mediated neutralization showed that Dugbe virus is intrinsically sensitive to the effects of complement and supports the explanation given above for the poor enhancement of IgG-mediated neutralization. It is interesting that high concentrations of IgM fail to bind complement as effectively as lower concentrations.

Extrapolating from electron microscope studies of the IgM neutralization of influenza virus (Armstrong et al., 1990), we believe this is because at high concentration IgM molecules bind in a planar rather than a crab-like configuration with respect to the surface of the virus, in a manner resembling dinner plates stacked in a plate rack. Complement-binding sites at the junction of the C4H2 and C4H3 domains are not exposed until IgM adopts the crab conformation (Feinstein et al., 1986) and there is thus no complement-enhanced neutralization.

References


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