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Roles of Immunoglobulin Valency and the Heavy-Chain Constant Domain in Antibody-Mediated Downregulation of Sindbis Virus Replication in Persistently Infected Neurons

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Clearance of infectious Sindbis virus from neurons is mediated by antibody to the E2 glycoprotein. Properties of the antibody important for downregulation of Sindbis virus replication are unknown. Immunoglobulin isotypes and valency determine many biological properties of antibodies. An immunoglobulin G1 (IgG1) isotype switch mutant and $F(ab')_2$ and Fab fragments of IgG3 monoclonal antibody 209 were prepared and tested for clearance of infectious virus from persistently infected rat dorsal root ganglion neurons in vitro. IgG1, IgG3, and IgG3-derived $F(ab')_2$ fragments were similarly efficacious, while IgG3-derived Fab fragments had no effect on virus replication. Cross-linking of Fab with secondary antibodies restored antiviral activity. Therefore, we found no evidence that IgG subclass plays a role in control of intracellular Sindbis virus replication. However, bivalency appears to be crucial for the ability of E2-specific IgG molecules to mediate clearance of infectious virus from neuron cells, suggesting that cross-linking of E2 molecules is essential.

Alphaviruses such as eastern and western equine encephalitis viruses are important causes of mosquito-borne encephalitis in the Americas (20). The prototype alphavirus, Sindbis virus (SV), causes acute encephalomyelitis in mice and serves as a useful animal model for studies of the pathogenesis of this disease (6). The primary cell in the nervous system that is infected is the neuron (8). Three- to four-week-old mice infected with the prototype AR339 strain of SV clear infectious virus from the nervous system within 7 to 8 days and recover uneventfully (9). Immunodeficient *scid* mice infected with the same strain of SV develop persistent nonlethal central nervous system infection (12). Infectious virus can be cleared by passive transfer of immune serum but not immune T cells (12). Studies of immunologically normal mice recovering from SV-induced encephalitis show infiltration of antibody-producing B cells into the brain beginning 3 to 4 days after infection and an increasing influx of antibody-secreting cells over the subsequent 1 to 2 weeks coincident with virus clearance (24, 25). Cells secreting SV-specific antibody remain in the brains of infected mice for at least a year after recovery (26). Persistence of antibody-secreting cells is postulated to be related to the persistence of SV RNA in brain cells after clearance of infectious virus and the need for continuous downregulation of virus replication (11, 26).

The antigenic specificity of the antiviral antibody that is responsible for virus clearance has been studied with monoclonal antibodies (MAbs) that recognize individual SV proteins. There are three proteins in the virion: a capsid protein that surrounds the RNA and two glycoproteins, E1 and E2, that form a heterodimer in the envelope of the virus. E2 is the major attachment protein and contains the epitopes that elicit the most potent neutralizing antibodies (4). E1 has a fusion peptide and is the major protein effecting entry of the virus into host cells (27). Both E1 and E2 are expressed on the surface of infected cells. MAbs to two different neutralizing epitopes on the E2 glycoprotein are effective in downregulating intracellular SV replication in persistently infected *scid* mice (12). The same MAbs are effective in clearing infectious SV from persistently infected cultures of rat dorsal root ganglion (DRG) neurons in vitro (12). The MAbs previously identified as capable of controlling SV replication in neurons in vivo or in vitro were of the immunoglobulin G (IgG) isotype (12).

MAb 209, an IgG3 antibody specific for the E2c epitope on the E2 glycoprotein, is particularly effective in controlling infection (12, 17). The biological functions of this MAb include neutralization of virus infectivity, inhibition of hemagglutination, limited complement-mediated lysis of SV-infected cells, and prevention of fatal encephalitis when given either before or after infection with a virulent strain of SV (17, 22, 23). IgG3 MAbs against bacterial polysaccharides are superior in binding to bacteria, in fixation of complement on the bacterial surface, and in opsonization of bacteria for phagocytosis compared with antibodies with the same variable regions that belong to different IgG subclasses (2, 21). Like polysaccharides, viruses and cells infected with viruses that bud from the plasma membrane have repetitive antigenic determinants on the surface. Therefore, it is possible that biologic functions of antiviral antibodies are also subclass dependent.

To determine whether IgG subclass affects the ability of SV antibodies to clear infectious virus from neurons, we have used the technique of sequential sublining (2, 18) to generate a mouse switch variant hybridoma producing an IgG1 MAb (G5)

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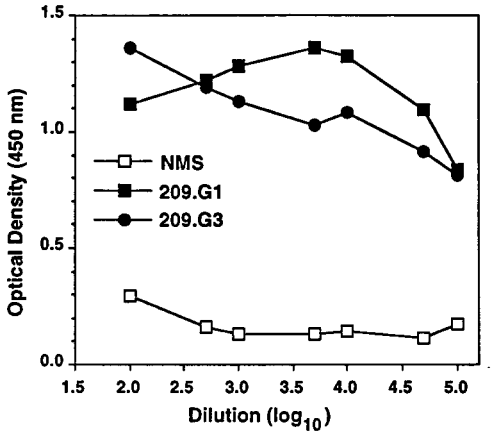

FIG. 1. EIA analysis of the binding of E2-specific MAb 209.G1 and MAb 209.G3 to SV. Normal mouse serum (NMS) was the control. Bound Ig was detected with peroxidase-conjugated rabbit anti-mouse Ig.

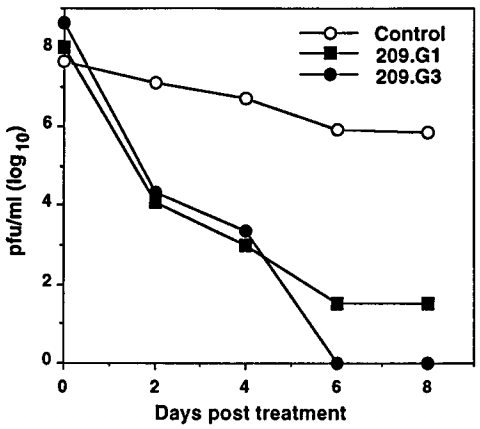

FIG. 2. Clearance of SV from persistently infected DRG neurons by E2-specific MAbs 209.G1 and 209.G3. Two days after infection, MAb was added at a dilution of 1:20 and incubated for 1.5 h. The medium containing MAb was removed, the culture was washed, and medium free of MAb replaced the MAb-containing medium. Control cultures were treated identically but did not contain MAb. The amounts of virus in supernatant fluids were determined by plaque formation on N18 mouse neuroblastoma cells (1).

expressing variable domains identical to those of the parental IgG3 MAb 209. Briefly, 209.G3 hybridoma cells were grown to high density and then plated at a concentration of 1,000 cells per well in a 96-well plate. Possible switch variants were identified with enzyme immunoassay (EIA) plates coated with goat anti-mouse IgG subclass-specific antibodies (Southern Biotech, Birmingham, Ala.) to which the hybridoma supernatant fluids were added. Goat anti-mouse IgG3 or IgG1 alkaline-phosphatase conjugates were then added, and the EIA was performed according to standard procedures. Positive wells were subcloned three times. Both monoclonal and polyclonal antisubclass antibodies were subsequently used to confirm subclass. Several IgG1 switch mutants were identified, and MAb G5 (209.G1) was used for subsequent studies. These antibodies (209.G1 and 209.G3) are thus directed to identical epitopes on the SV E2 glycoprotein but differ in subclass.

Ascites fluids containing the 209.G1 and 209.G3 MAbs were tested for binding to the recombinant TE strain of SV (14) in an EIA (Fig. 1). The EIA was performed essentially as previously described (22) with plates coated with 0.3 μg of gradient-purified SV, horseradish peroxidase-conjugated anti-mouse Ig (Dako, Santa Barbara, Calif.), and orthophenyl diamine as substrate. Plates were read at 450 mm (Titertek Multiscan; Flow Laboratories, McLean, Va.). 209.G1 and 209.G3 bound similarly to SV-coated wells.

The ability of the MAbs to downregulate SV replication was assessed in vitro by treatment of persistently infected DRG neurons (Fig. 2). DRGs were explanted from 18-day-old rat embryos and cultured in nerve growth factor, as previously described, for 5 to 6 weeks before infection (12, 13). DRG neurons matured to this extent develop a persistent infection after exposure to 10^3 PFU of the TE strain of SV (12, 13). Forty-eight hours after infection, DRG neuron cultures were treated with a 1:20 dilution of 209.G1 or 209.G3 MAbs for 1.5 h at 37°C. The MAb-containing medium was removed, the cultures were washed twice, and culture medium without MAb was added. Virus clearance was assessed over the next week by measuring PFU in the supernatant fluids (Fig. 2). 209.G1 and 209.G3 MAbs cleared infectious SV with similar efficiency.

To determine if the Fc portion of the IgG molecule was of any importance for clearance, $F(ab')_2$ fragments were prepared by digestion of purified MAb 209.G3 with immobilized pepsin (Pierce, Rockford, Ill.) at 37°C for 1 h. $F(ab')_2$ fragments were purified by passage over a protein A/G column (Pierce) and assessed for ability to clear virus (Fig. 3). $F(ab')_2$ was as efficient as intact IgG in downregulating SV infection in persistently infected DRG neurons.

To determine if bivalency of the antibody molecules was important for clearance, Fab fragments were prepared by digestion of purified MAb 209.G3 with immobilized papain (Pierce) at 37°C for 4 h. Fab fragments were separated from Fc fragments and undigested IgG by passage over a protein A/G column. Fab fragments had no effect on SV replication in DRG cells (Fig. 3). To confirm that the preparation of Fab fragments had not affected the ability of the antibody to bind viral antigen, purified intact IgG, $F(ab')_2$ fragments, and Fab fragments were tested in EIA. Similar optical density EIA readings were obtained for comparable amounts of Fab. To determine if the ability of Fab fragments to clear infection could be restored by cross-linking the Fab fragments, antibodies specific for Fab, the H chain, and the κ chain of the antibody molecule were added and virus clearance was assessed (Fig. 4). Cross-linking Fab fragments with any of these antisera restored the ability of SV E2-specific Fab to downregulate SV

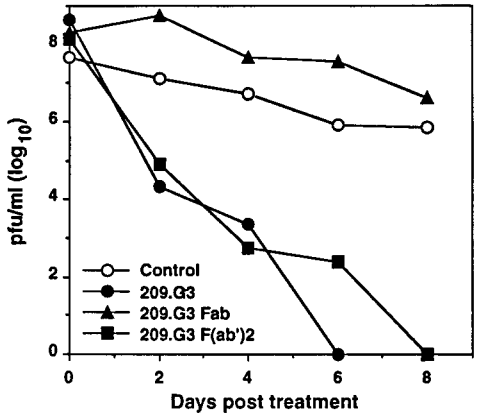

FIG. 3. Clearance of SV from persistently infected DRG neurons by $F(ab')_2$ and Fab fragments of MAb 209.G3. Cultures were treated with 30 μg of antibody fragment protein per ml as described in the legend to Fig. 2.

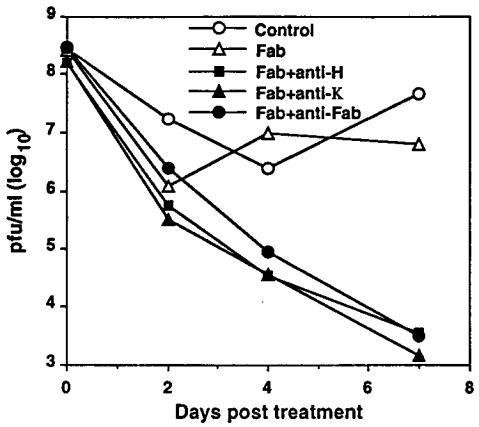

FIG. 4. Clearance of SV from persistently infected DRG neurons by Fab fragments of MAb 209.G3. The effects of Fab (30 μg/ml) used alone and Fab cross-linked by antibody specific for murine Fab, H chain, or κ chain were assessed as described in the legend to Fig. 2.

replication in persistently infected neurons. However, cross-linked Fab was not quite as effective as intact antibody since virus could still be detected 7 days after treatment.

The role of Ig subclass in the biologic activities of antiviral antibodies has received little attention. In many viral infections, clearance of virus from tissue appears to be dependent on elimination of infected cells by cytotoxic T cells and antibody serves primarily to neutralize the infectivity of extracellular virus. However, for some groups of viruses (e.g., alphaviruses and enteroviruses) and for some tissues (e.g., the nervous system) antibody has an important role in controlling virus replication (5, 7, 16). Previous studies have shown that antibody to the E2 glycoprotein is capable of eliminating infectious SV from neurons, but the mechanism remains unclear (12). In these studies, we have further defined the biologic properties of antibody important for control of SV replication. Using isotype switch mutants, we have shown that, for antibodies with the same specificities and abilities to bind virus, IgG1 and IgG3 are equally effective at inhibiting virus replication in persistently infected neurons. Further study showed that inhibition of virus replication does not require the Fc portion of antibody but does require bivalency of the Fab portions of the molecule, suggesting the necessity for cross-linking of antigen.

Previously identified MAbs that effect clearance of SV from persistently infected neurons in vivo and in vitro have belonged to the IgG2a and IgG3 subclasses (12). The current study has shown that IgG1 MAb is also effective in downregulating SV replication. The subclass of serum IgG antibodies produced most abundantly by mice after intranasal infection with a variety of viruses, including SV, is IgG2a (3). This shift is dependent on virus replication and appears to reflect the cytokines induced during the immune response to viral infection, particularly gamma interferon (15, 19). Studies of the Ig isotypes produced by local SV-specific antibody-producing cells after intracerebral inoculation of BALB/c mice with SV have shown that IgM-secreting cells appear in the brain early after infection (days 3 to 4) and are followed approximately 1 week later by the appearance of SV-specific IgA-, IgG1-, IgG2a-, IgG2b-, and IgG3-secreting cells. For most Ig isotypes, the frequency of an SV-specific antibody isotype in the brain is in proportion to the frequency of the isotype in the spleen and lymph nodes, with the largest number of cells producing IgG2a and the fewest producing IgG3 (24). This suggests that isotype switching is usually induced in the periphery. However, IgA and IgG1 are disproportionately increased in the brain compared with spleen and cervical lymph nodes, suggesting that local factors influence B-cell entry or isotype switching in the central nervous system (24). The importance of this selection is not known but may reflect the early and prolonged production of Th2-type cytokines in the central nervous system in response to SV infection (28).

The mechanism by which antibody downregulates SV replication in neurons is unclear. Antibody may be acting by binding virus proteins on the surface of the infected cell or by entering the cell either alone or in association with viral proteins. These studies demonstrate that bivalent antibody is essential and suggest that cross-linking of the E2 glycoprotein is important for antibody-mediated suppression of virus replication. This may suggest that transmembrane signaling is involved in altering cellular support of virus replication, since cross-linking of cell surface receptors by ligands is often necessary for signal transduction. Alternatively, cross-linking of the E2 glycoprotein may alter other cellular functions controlled at the plasma membrane or may promote antibody uptake by the infected cell (10), leading to a direct effect on virus replication.

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