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Characterization of a Monoclonal Antibody Directed to the Surface of MA104 Cells That Blocks the Infectivity of Rotaviruses

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Rhesus rotavirus (RRV) binds to sialic acid residues on the surface of target cells, and treatment of these cells with neuraminidase greatly reduces virus binding with the consequent reduction of infectivity. Variants that can efficiently infect neuraminidase-treated cells have been isolated, indicating that attachment to sialic acid is not an essential step for animal rotaviruses to infect cells. To identify and characterize the neuraminidase-resistant receptor for rotaviruses, we have isolated a hybridoma that secrets a monoclonal antibody (MAb) (2D9) that specifically blocks the infectivity of wild-type (wt) RRV and of its sialic acid-independent variant nar3, in untreated as well as in neuraminidase-treated cells. The infectivity of a human rotavirus was also inhibited, although to a lesser extent. MAb 2D9 blocks the binding of the variant to MA104 cells, while not affecting the binding of wt RRV; in addition, this MAb blocked the attachment of a recombinant glutathione *S*-transferase (GST)–VP5 fusion protein, but did not affect the binding of GST–VP8. Altogether these results suggest that MAb 2D9 is directed to the neuraminidase-resistant receptor. This receptor seems to mediate the direct attachment of the variant to the cell, through VP5, while the receptor is used by wt RRV for a secondary interaction, after its initial binding to sialic acid, through VP8. MAb 2D9 interacts specifically with the cell surface by indirect immunofluorescence, immunoelectron microscopy, and FACS. By a solid-phase immunoisolation technique, MAb 2D9 was found to react with three proteins of ca. 47, 55, and 220 kDa, which might form a complex.

INTRODUCTION

The initial event in the interaction of a virus with the host cell is the attachment of the virus to receptors in the cell membrane. Attachment is mediated by a variety of moieties on host cells ranging from the general, such as sialic acid (SA), to specific extracellular integral membrane proteins. Any cell surface entity that mediates this attachment is defined as a viral receptor; consequently the expression of the receptor on specific cells or tissues in the whole host is a major determinant of the route of virus entry into the host, the pattern of virus spread, and the resulting pathogenesis (Haywood, 1994). An understanding of the mechanisms of viral cell attachment may provide insight into the tissue tropism of a particular virus, as well as potential treatments for viral diseases.

Rotaviruses are the leading cause of morbidity and mortality, due to acute gastroenteritis, in children under 2 years of age (Kapikian and Chanock, 1996). These viruses, members of the family *Reoviridae,* are nonenveloped and possess a genome of 11 segments of dsRNA contained in a triple-layered protein capsid. The outermost layer is composed of two proteins, VP4 and VP7. The smooth external surface of the virus is made up of 780 copies of the glycoprotein VP7, while 60 spike-like structures, formed by dimers of VP4, extend about 12 nm from the VP7 surface (Estes, 1996; Prasad *et al.,* 1990).

VP4 has essential functions in the virus life cycle, including receptor binding and cell penetration (Estes and Cohen, 1989). The properties of this protein are therefore important determinants of host range, virulence, and induction of protective immunity. The infectivity of rotaviruses is dependent on trypsin treatment of the virus, and this proteolytic treatment results in the specific cleavage of VP4 to polypeptides VP8 and VP5 (Arias *et al.,* 1996; Espejo *et al.,* 1981; Estes *et al.,* 1981). The cleavage of VP4 does not affect cell binding (Clark *et al.,* 1981; Fukuhara *et al.,* 1988; Kaljot *et al.,* 1988) and has been associated with the entry of the virus by direct plasma membrane penetration (Kaljot *et al.,* 1988; Nandi *et al.,* 1992; Ruiz *et al.,* 1994). Recently, it was shown that the virus uses both VP8 and VP5 proteins to bind to the surface of MA104 cells in a SA-dependent and -independent manner, respectively (Zárate *et al.,* 2000). The role of VP7 during the early interactions of the virus with the cell is not clear, although it has been shown that it can modulate some

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of the VP4-mediated virus phenotypes, including receptor binding (Méndez *et al.,* 1996).

Rotavirus infection is highly restricted *in vivo* to the mature villus tip cells of the small intestine. The infection *in vitro* is also restricted, being most permissive in a variety of epithelial cell lines of renal and intestinal origin (Kapikian and Chanock, 1996). The high degree of selectivity of these viruses suggests the presence of specific receptors in the surface of susceptible cells, which might be at least one of the factors responsible for determining the virus tropism.

Some rotaviruses of animal origin bind to the cell surface through a SA-containing cell receptor (Ciarlet and Estes, 1999; Fukudome *et al.,* 1989; Keljo and Smith, 1988; Méndez *et al.,* 1993). We have isolated variants from a SA-dependent rhesus rotavirus (RRV) that no longer depend on the presence of SA to bind and thus to infect the cell. The characterization of these variants showed that binding to SA is not an essential step for infection of cells by SA-dependent animal rotaviruses; it also showed that the initial interaction with SA can be superseded by an interaction with a secondary receptor [neuraminidase (NA)-resistant] that might be responsible, at least in part, for the tropism of these viruses (Méndez *et al.,* 1993). In contrast to animal rotaviruses, most, if not all, human rotaviruses do not require SA to infect the cells (Ciarlet and Estes, 1999; Keljo and Smith, 1988).

Based on competition experiments among strains of human and animal origin, together with a SA-independent RRV variant, it was established that the interaction of rotavirus with its host cell is a multistep process in which a SA-containing component, a SA-independent molecule, and probably a third unidentified molecule are involved (Méndez *et al.,* 1999).

Recently, it was reported that VP4 and VP7 contain integrin ligand sequences, and peptides containing these ligand motifs and monoclonal antibodies (MAbs) to the respective integrins inhibited the infectivity of the simian rotavirus strain SA11 and of the human rotavirus strain RV5, implicating $\alpha 2\beta 1$ and $\alpha 4\beta 1$ integrins in the cell entry of these viruses (Coulson *et al.,* 1997; Hewish *et al.,* 2000).

To identify other possible cell surface molecules involved in the process of rotavirus entry we have generated MAbs directed to MA104 cells. In this work we report the isolation and characterization of a MAb directed to the surface of these cells that is able to specifically prevent the infectivity of the SA-dependent RRV and of its NA-resistant variant nar3.

RESULTS

Isolation and screening of hybridomas. We were interested in isolating a MAb directed to the NA-resistant rotavirus receptor. Thus, hybridomas were prepared from mice immunized with whole NA-treated MA104 cells. Approximately 2500 hybridoma culture supernatants were screened for their ability to block the infectivity of the NA-resistant rotavirus variant nar3 in an immunoperoxidase focus reduction assay (Arias *et al.,* 1987). One hybridoma that efficiently inhibited nar3 infection was subcloned three times by limiting dilution until a stable antibody-producing clone was isolated. The MAb secreted by this clone, 2D9, was isotyped as IgM. In the experiments described next, MAb 2D9 was used as a purified fraction from ascites fluid.

Virus specificity of monoclonal antibody 2D9. The specificity of MAb 2D9 was determined by its ability to block the infectivity of the wt virus RRV, its SA-independent variant nar3, and the human rotavirus strain Wa. Preincubation of MA104 cells with different dilutions of MAb 2D9 resulted in the reduction of the focus-forming units (FFU) by 75% for nar3, by 60% for RRV, and by 25% for Wa. This reduction in infectivity was dependent on the concentration of MAb added to the cell monolayer. Preincubation of the cells with a commercial, control mouse IgM did not affect the infectivity of any of these viruses (Fig. 1).

Given that the hybridoma secreting 2D9 was obtained by immunizing mice with MA104 cells treated with NA, we tested the ability of the MAb to prevent the infection of the same three virus strains on cells treated with this enzyme. Given the low level of infectivity of RRV under these conditions (Méndez *et al.,* 1993), the amount of RRV used in this experiment was increased sixfold to maintain a similar number of FFU per well. The blocking activity of MAb 2D9 in NA-treated cells for the three virus strains was essentially that observed in untreated cells (Fig. 1).

To rule out the possibility that MAb 2D9 protected MA104 cells by nonspecific masking of the cell surface, the ability of this antibody to protect MA104 cells against the infection of two other nonenveloped viruses was evaluated. Figure 2 shows that 2D9 did not affect the infectivity of either poliovirus type 3 or reovirus type 1 in MA104 cells.

Altogether these results indicate that MAb 2D9 is able to specifically block the infectivity of rotaviruses RRV and nar3, and to a lesser extent that of the human strain Wa, and that, most likely, the epitope recognized by this MAb does not involve SA.

MAb 2D9 prevents the binding of nar3 but not that of RRV. Since MAb 2D9 was able to significantly block the infectivity of both RRV and its SA-independent variant nar3 in MA104 cells, we next asked whether the inhibition of infectivity was due to the prevention of binding of the viruses to the cell surface. For this, we used a nonradioactive binding assay in which the cell-bound virus was detected by a specific enzyme-linked immunosorbent assay (ELISA) (Zárate *et al.,* 2000). In this experiment, a suspension of MA104 cells was preincu-

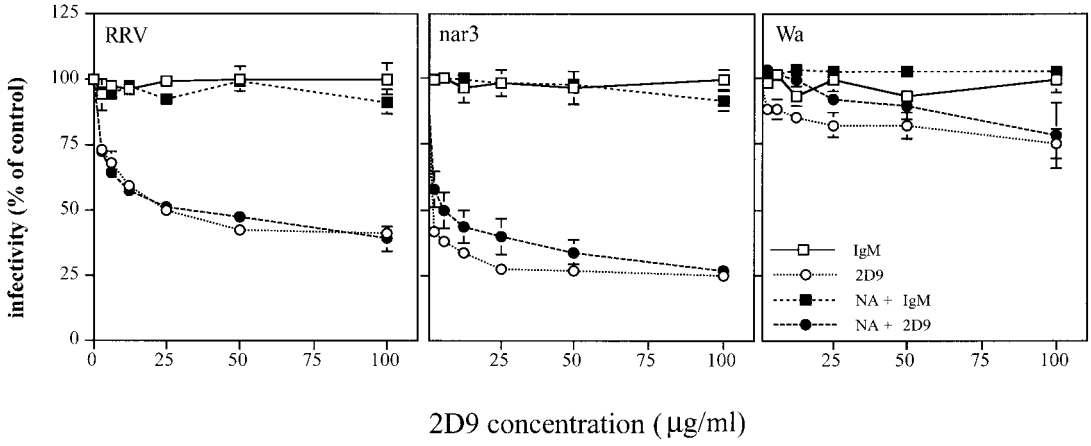

FIG. 1. MAb 2D9 blocks rotavirus infectivity in neuraminidase-treated and untreated MA104 cells. The indicated concentrations of purified 2D9 or a control IgM were added to monolayers of MA104 in 96-well plates that had been either treated (solid symbols) or not (open symbols) with NA for 1 h at 37°C. After incubation with the antibodies, the cells were washed twice with PBS and then 2×10^3 FFU of RRV, nar3, or Wa viruses was added per well to both treated and untreated cells, with the exception of RRV in NA-treated cells, where 1.2×10^4 FFU was used, since the infectivity of RRV under these conditions is reduced five- to six-fold. After 1 h of adsorption at 37°C, the viral inoculum was removed, and the infection was left to proceed for 16 h at 37°C, at which time the cells were fixed and immunostained, as described under Materials and Methods. Data are expressed as a percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. The arithmetic mean ± standard error from five independent experiments performed in duplicate is shown.

bated with different dilutions of either 2D9 or a control mouse IgM for 1 h at 4°C; after removal of the excess antibody the cells were incubated with a fixed amount of either RRV or nar3. We found that while the preincubation of the cells with 2D9 did not affect the binding of wt RRV, the binding of nar3 was reduced in a concentration-dependent manner up to about 25% of that of the control cells (Fig. 3). The binding of the human strain Wa, in the presence of either 2D9 or IgM, was not significantly affected (not shown).

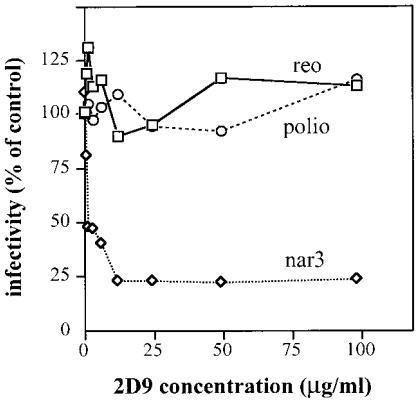

FIG. 2. Blocking specificity of MAb 2D9. The indicated concentrations of purified 2D9 were added to monolayers of MA104 in 96-well plates for 1 h at 37°C and washed twice with PBS and then, approximately 2×10^3 FFU of nar3, reovirus type 1, or poliovirus type 3 was added per well. After 1 h of adsorption at 37°C, the viral inoculum was removed, and the infection was left to proceed for 16 h at 37°C, at which time the cells were fixed and immunostained, as described under Materials and Methods. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. The arithmetic mean ± standard error from two independent experiments is shown.

We have recently shown that RRV initially binds to the cell surface through the VP8 domain of VP4, while nar3 binds through VP5 (Zárate *et al.,* 2000). Since 2D9 is able to inhibit the binding of nar3 but not that of RRV, we next asked whether this MAb was able to differentially prevent the binding of recombinant VP5 and VP8 proteins, which have been previously shown to bind specifically to the cell surface (Zárate *et al.,* 2000). For this, a suspension of MA104 cells was preincubated with MAb 2D9, or a mouse IgM as control, and then a constant amount of either glutathione *S*-transferase (GST)–VP5 or GST–VP8 fusion proteins produced in bacteria was added. The cell-bound recombinant proteins were detected by ELISA as previously described (Zárate *et al.,* 2000). Figure 4 shows that while the binding of GST–VP8 was not affected by preincubation of the cells with either 2D9 or the IgM control, the binding of GST–VP5 was reduced to about 30% of the control value with no antibody. Altogether these results suggest that MAb 2D9 might be recognizing the same molecule used by the NA-resistant variant nar3 to interact with the cell, through its VP5 protein.

The epitope recognized by 2D9 localizes to the surface of MA104 cells. The cellular localization of the epitope recognized by MAb 2D9 was investigated by immunoelectron microscopy of MA104 cells. Figure 5A shows that MAb 2D9 labeled primarily the surface of the cells as judged by the deposition of the peroxidase substrate, DAB, in the plasma membrane, whereas in the control cells, incubated with a mouse IgM, this pattern was not observed (Fig. 5B). The DAB substrate stained only one face of the cells due to the fact that fixation and immunostaining were done while the cells were still bound to the surface of the flask.

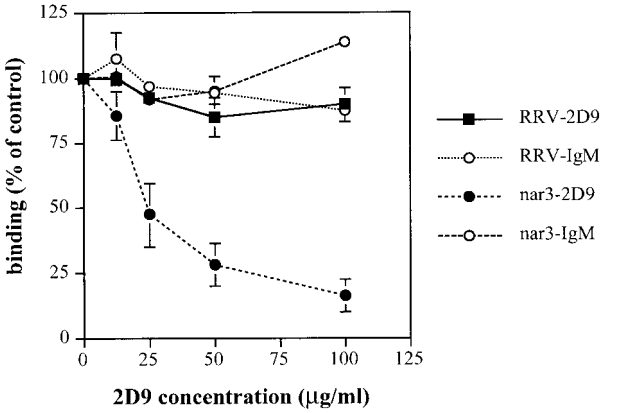

FIG. 3. Binding of RRV and nar3 viruses to cells in the presence of MAb 2D9. The indicated amounts of MAb 2D9 or a control IgM were preincubated with 5×10^4 MA104 cells in suspension for 1 h at 4°C. The excess, unbound antibody was removed, and then 300 ng of either RRV or nar3 purified virus particles was added, and the mixture was further incubated for 1 h at 4°C. The amount of virus bound to cells was determined by an ELISA as described under Materials and Methods. Data are expressed as percentage of the virus binding obtained when the virus particles were preincubated with PBS as a control. The arithmetic mean ± standard error from two independent experiments performed in duplicate is shown.

The presence of the epitope recognized by 2D9 on the cell surface was further confirmed by indirect immunofluorescence of unpermeabilized MA104 cells. MAb 2D9 recognized an epitope that was distributed in a patched pattern on the surface of the cells, which was more clearly observed in the junction between cells (Fig. 5C). Control cells stained with an IgM did not show this pattern (Fig. 5D). Also, using flow cytometric analysis, we found that 2D9 was able to bind to the surface of MA104 cells (Fig. 5E), as predicted if directed to a virus receptor structure.

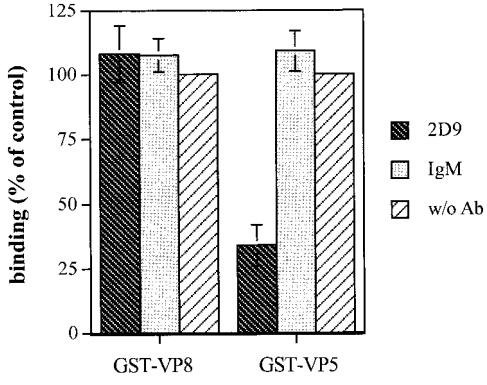

FIG. 4. Binding of recombinant proteins GST–VP8 and GST–VP5 to cells in the presence of MAb 2D9. MAb 2D9 or a control IgM (100 μg/ml) was preincubated with 5×10^4 MA104 cells in suspension for 1 h at 4°C. The excess, unbound antibody was removed, and then 1.5 μg of affinity-purified GST–VP5 or GST–VP8 was added, and the mixture was further incubated for 1 h at 4°C. The amount of cell-bound protein was determined by ELISA as described under Materials and Methods. Data are expressed as percentage of the recombinant protein binding obtained when the fusion proteins were preincubated with PBS as a control (w/o MAb). The arithmetic mean ± standard error from two independent experiments performed in duplicate is shown.

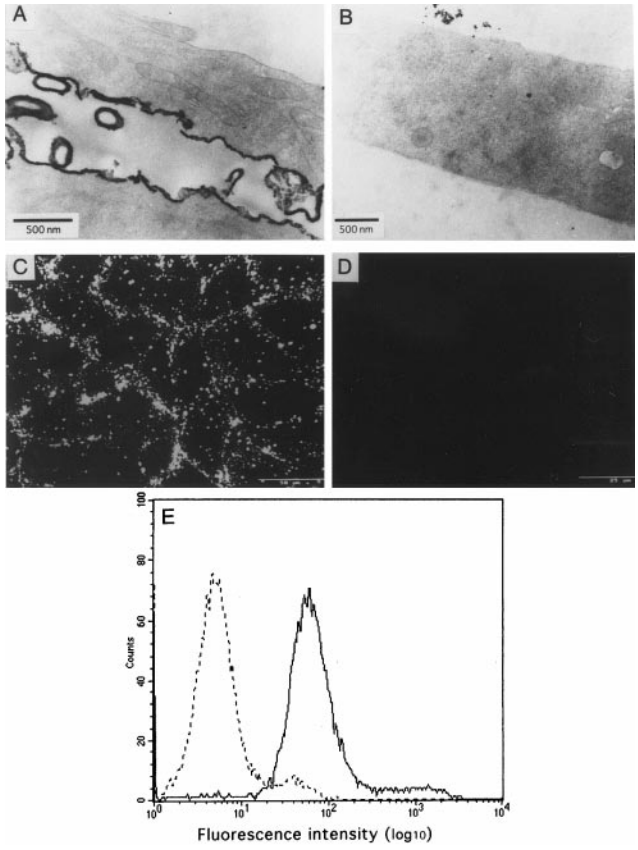

FIG. 5. Monoclonal antibody 2D9 interacts with the surface of MA104 cells. (A and B) Electron micrographs of immunoperoxidase staining of MA104 cells. Immunomarking was performed directly on MA104 cell monolayers, which were incubated with (A) MAb 2D9 (10 μg/ml) or with (B) a control IgM (10 μg/ml) and then stained with peroxidase-conjugated goat anti-mouse IgM and DAB as substrate. The cells were then detached from the flasks, postfixed with 1% OsO_4, and processed for electron microscopy as detailed under Materials and Methods. (C and D) Indirect immunofluorescence of MA104 cells. Cells were incubated with MAb 2D9 (10 μg/ml; C) or with a control IgM (10 μg/ml; D) and then stained with affinity-purified goat anti-mouse IgM antibodies conjugated to fluorescein isothiocyanate. (E) Binding of MAb 2D9 to MA104 cells as determined by flow cytometry. Cells were incubated either with MAb 2D9 (20 μg/ml, solid line) or with IgM (20 μg/ml, dashed line) and stained with affinity-purified goat anti-mouse IgM antibodies conjugated to fluorescein isothiocyanate. The amount of IgM bound was assayed by flow cytometry as described under Materials and Methods.

Solid-phase immunoisolation of MA104 cell proteins by MAb 2D9. Attempts to immunoprecipitate proteins from a cell lysate with MAb 2D9 were unsuccessful. Thus, to determine the cell surface protein to which the MAb was directed, we performed a solid-phase immunoisolation technique (SPIT) assay, in which either MAb 2D9 or a control IgM were adsorbed to wells of an ELISA plate. Total MA104 cell lysates labeled metabolically with ^{35}S or with sulfo-NHS-biotin, which labels proteins exposed in the cell surface (since it is a membrane-impermeable

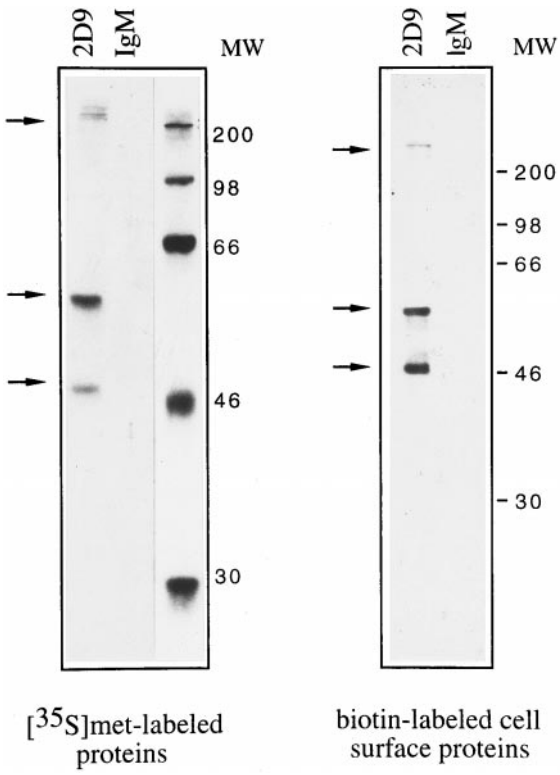

FIG. 6. Solid-phase immunoisolation of MA104 cell proteins by MAb 2D9. Cell lysates metabolically labeled with ^{35}S ([^{35}S]Met-labeled proteins) or with sulfo-NHS-biotin (biotin-labeled proteins) were added to antibody (2D9 or IgM)-coated wells. After incubation, the wells were washed, and the proteins that remained bound to the wells were released with Laemmli sample buffer. The samples were analyzed by SDS–PAGE and fluorography (for ^{35}S-labeled proteins) or by Western blot (for the biotin-labeled proteins) staining with streptavidin coupled to peroxidase, as indicated under Materials and Methods. MW, molecular weight markers.

reagent), were added to the antibody-coated wells. The wells were later extensively washed, and the proteins that remained bound were released with Laemmli sample buffer and analyzed by SDS–PAGE and fluorography (for ^{35}S-labeled proteins) or transferred to nitrocellulose (for the biotin-labeled proteins) and stained with streptavidin coupled to peroxidase (Fig. 6). It can be observed that MAb 2D9 captured from the total protein cell lysate a group of three proteins of ca. 47, 55, and 220 kDa, which were not recognized by the control IgM. These three proteins must be exposed on the surface of the cell, since the SPIT assay performed with the biotin-labeled polypeptides showed three proteins with the same molecular masses. Again, when the biotin-labeled lysate was incubated in a well coated with the control IgM, no biotinylated proteins were observed. These results suggest that MAb 2D9 interacts with a cell surface protein that might be forming a complex of at least three proteins or that the three proteins that are captured by 2D9 share a common epitope recognized by the antibody.

DISCUSSION

It has been known for some time that rotaviruses can interact with the surface of susceptible cells by at least two different mechanisms; some rotaviruses of animal origin attach to a sialic acid-containing molecule, while rotaviruses of human origin bind through a neuraminidase-resistant compound (Ciarlet and Estes, 1999; Mendez *et al.,* 1993). However, it has been recently shown that the initial interaction of animal rotaviruses with SA is not essential, since variants that bind and infect cells in a SA-independent manner have been isolated from the SA-dependent RRV (Ciarlet and Estes, 1999; Ludert *et al.,* 1996; Mendez *et al.,* 1993).

One of the strategies that has been successfully used to identify the cell receptors for viruses belonging to several different families has been the isolation of hybridomas that secrete MAbs directed to the surface of susceptible cells, which are able to block virus entry. The putative receptors for measles virus (Dunster *et al.,* 1994), Sindbis virus (Wang *et al.,* 1992), enterovirus 70 (Karnauchow *et al.,* 1996), echoviruses (Bergelson *et al.,* 1994), and vaccinia virus (Chang *et al.,* 1995), among other examples, have been identified following this strategy.

Following this approach, we isolated a hybridoma that secretes a MAb directed against the surface of NA-treated MA104 cells, a highly permissive cell line for rotavirus. This MAb (2D9), of IgM isotype, is able to specifically block the infectivity of both wt RRV and its variant nar3, and to a lesser extent, the infectivity of the human strain Wa. MAb 2D9 also blocks the infectivity of these viruses in NA-treated cells.

The antigen recognized by 2D9 is present on the surface of MA104 cells as judged by fluorescence-activated cell sorting (FACS) analysis, immunofluorescence, and immunoelectron microscopy. Using the first two methods we screened several cell lines for the presence of this antigen. We found that MAb 2D9 specifically recognized the surface of CV-1, CaCo2, and COS7 cells, all susceptible to rotavirus infection. However, this MAb also reacted with the surface of Hep-2, L, CHO, and BHK cells, which are much less susceptible to infection by rotaviruses (Espinosa *et al.,* unpublished results), indicating that the antigen recognized by 2D9 is not the only factor that determines the tropism of these viruses.

A number of glycoconjugates have been shown to bind to, and to block the infectivity of, SA-dependent animal rotavirus strains, and some of them have been suggested to play a role as possible receptors, like GM3 gangliosides in newborn piglet intestine (Rolsma *et al.,* 1998), GM1 in LLC-MK2 cells (Superti and Donelli, 1991), and 300 to 330-kDa glycoproteins in murine enterocytes (Bass *et al.,* 1991). More recently, it was reported that integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$ are involved in the entry of rotaviruses (Coulson *et al.,* 1997). The fact that the

epitope recognized by MAb 2D9 is present on the surface of CHO and L cells, which do not express $\alpha 2$ or $\alpha 4$ integrins (Hewish *et al.,* 2000; Zhang and Racaniello, 1997), and the fact that the reported molecular weight for these integrins does not coincide with that of the proteins detected by 2D9 suggest that this MAb recognizes a molecule different from integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$.

We have recently reported that wt RRV interacts initially with SA residues on the surface of MA104 cells, through the VP8 domain of its VP4 protein. We proposed that this initial interaction is subsequently followed by a second interaction of the VP5 domain of VP4 with a NA-resistant cell receptor. We also showed that the SA-independent variant nar3 is able to interact directly with the cell surface through VP5, obviating the first VP8–SA interaction (Zárate *et al.,* 2000). In this work we have found that MAb 2D9, despite inhibiting the infectivity of both wt RRV and nar3 viruses, competes only the attachment of the variant, suggesting that it blocks the infectivity of the wt virus at a postattachment step, in agreement with our previous observations. In accordance with these results, we found that 2D9 blocks the binding of the GST–VP5 fusion protein, while it does not affect the attachment of the GST–VP8 recombinant polypeptide (Fig. 4). Taken together, these results suggest that the epitope recognized by 2D9 is independent of sialic acid and is probably present in the cell receptor that interacts with VP5 or in a molecule closely associated with it.

Based on an infection competition assay, designed to detect competition for cell surface molecules at both attachment and postattachment steps (Mendez *et al.,* 1999), we found that the human strain Wa efficiently competed the infectivity of the variant nar3 both in untreated and in NA-treated cells. This competition was nonreciprocal since nar3 did not compete the infectivity of Wa. The fact that the competition between the two NA-resistant strains, nar3 and Wa, was not reciprocal indicates that they bind to different molecules. In addition, the SA-dependence phenotype clearly differentiates RRV from nar3 and Wa, suggesting the existence of at least three cellular structures involved in rotavirus cell infection, with at least one being shared by human, SA-dependent animal, and NA-resistant variant strains. The antigen recognized by 2D9 on the surface of the cell might represent one of these interactions.

The list of viruses that have more than one interaction with the cell surface during cell entry is growing (Haywood, 1994; Norkin, 1995), indicating that our initial view that the virus–receptor interactions resembled those of simple ligands with their receptors was not completely true. It is becoming more and more apparent that the interaction of a virus with the surface of its host cell, which ultimately leads to the entry of the virus into the cell's cytoplasm, is a dynamic process in which more than one virus–cell interaction often takes place and in which conformational changes of both viral and host cell proteins might occur (Haywood, 1994; Norkin, 1995; Olson *et al.,* 1999). Furthermore, it has become apparent that some viruses may use more than one receptor to gain access into their host cells (Baranowski *et al.,* 2000; Thoulouze *et al.,* 1998; Tufano, 1997).

Despite our efforts, it was not possible either to immunoprecipitate or to detect by Western blots the molecule(s) recognized by MAb 2D9. However, by a solid-phase immunoisolation technique, 2D9 specifically interacted with three proteins that were present on the surface of the cell, as judged by the fact that they could be labeled with a reagent that is impermeable to the cell membrane. Although it is possible that these three proteins contain a common epitope recognized by MAb 2D9, it seems more likely that the proteins might be forming a complex, which remains as such under the conditions used to lyse the cells, with only one of them being recognized by 2D9. The identity of these proteins is currently under investigation.

The fact that neither 2D9 nor any of the anti-integrin antibodies that have been assayed so far are able to completely block the infectivity of rotaviruses [this work and Coulson *et al.,* (1997)], together with the finding that MAb 2D9 reacts with the surface of cells that are poorly susceptible to rotavirus, supports our idea that the interaction of rotavirus with its host cell is a multistep process (Mendez *et al.,* 1999) that involves interactions with several different molecules on the cell surface. The exquisite tropism of rotaviruses, which *in vivo* infect only a very narrow set of cells in the intestine, might be explained if only this type of cell possesses on its surface the appropriate combination of the required receptor molecules, which might be present individually in many different cell types. It remains to be determined how many cell surface molecules are involved in the complex process of rotavirus cell attachment and penetration and the role they may play.

MATERIALS AND METHODS

Cells, viruses, and monoclonal antibodies. MA104 and L929 cells (L cells) were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Rotavirus strains RRV and Wa were originally obtained from H. B. Greenberg (Stanford University, Stanford, CA), and rotavirus variant nar3 has been described previously (Méndez *et al.,* 1993). RRV, Wa, and nar3 viruses were propagated in MA104 cells as previously described (Espejo *et al.,* 1980). Reovirus serotype 1 was obtained from C. Ramos (Instituto Nacional de Salud Pública, Cuernavaca, Morelos, México) and was grown in L cells as described (Cuadras *et al.,* 1997). Poliovirus type 3 was obtained from R. M. del Angel (CINVESTAV, Mexico D.F., Mexico) and was grown in MA104 cells. Rabbit polyclonal antibody against reovirus type 1 was

kindly provided by T. Dermody (Vanderbilt Medical School, Nashville, TN).

To prepare purified virus, virus-infected cells were harvested after complete cytopathic effect was attained, the cell lysate was frozen–thawed twice, and the virus was pelleted by centrifugation for 60 min at 25,000 rpm at 4°C in the SW28 rotor (Beckman). The virus pellet was resuspended in TNC buffer [10 mM Tris–HCl (pH 7.5), 140 mM NaCl, 10 mM $CaCl_2$], extracted with Freon, and subjected to isopicnic centrifugation in CsCl as previously described (Espejo *et al.,* 1981). The protein content of the purified triple-layered particles was determined by the Bradford protein assay (Bio-Rad).

The infectious titer of the viral preparations was obtained by an immunoperoxidase focus assay in MA104 cells grown in 96-well tissue culture plates, as described (Arias *et al.,* 1987). Titers are expressed as focus-forming units per milliliter. When indicated, cells were treated with 20 mU/ml of NA from *Arthrobacter ureafaciens* (Sigma Chemical Co.) for 1 h at 37°C. After two washes with PBS [0.2 M NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 0.8 mM Na_2HPO_4 (pH 7.4)], the cells were infected as described (Méndez *et al.,* 1993).

Production of monoclonal antibodies. Confluent monolayers of MA104 cells were washed and brought into a single-cell suspension by incubation with 5 mM EDTA in PBS for 10 min at 37°C and dispersed by gentle pipetting. The cell suspensions were centrifuged at 1000 rpm for 1 min at 4°C, washed, resuspended into PBS, and treated with 20 mU of *A. ureafaciens* neuraminidase (Sigma Chemical Co.) for 1 h at 37°C with gentle rocking. The cells were then washed twice with PBS and resuspended in PBS and their concentration was determined with a hemocytometer. Inbred female BALB/c mice (8 weeks old) were immunized intraperitoneally with 7.5×10^6 whole MA104 cells, pretreated with NA, at 2-week intervals (four times total). Antibody production was monitored by measuring the ability of the sera to block the infectivity of nar3 and RRV viruses (see above). Two days prior to cell fusion, mice were primed by tail vein injection of 1×10^7 whole MA104 cells in PBS, pretreated with NA. Spleens from immunized mice were fused with FOX myeloma cells essentially as described by Padilla-Noriega *et al.,* (1993). Supernatants from viable hybridoma cultures were screened for the presence of antibodies that blocked the infectivity of nar3 by an immunoperoxidase focus reduction assay, in MA104 cells grown in 96-well tissue culture plates, as previously described (Arias *et al.,* 1987). Hybridomas producing blocking antibodies were cloned three times by limiting dilution and their supernatants were retested.

The blocking activity of one hybridoma, named 2D9, was confirmed after repeated tests. This MAb was shown to be of the IgM class by double immunodiffusion using isotype-specific antibodies (ICN). For production of ascites fluid containing MAb 2D9, 8-week BALB/c female mice, primed intraperitoneally with 200 μl of Pristane, were inoculated with the 2D9 hybridoma. The ascitic fluid was collected 2 weeks after the hybridoma immunization, and the IgM fraction was obtained by dialysis of the ascitic fluid in distilled water as described (Andrew *et al.,* 1997). The protein content of the purified IgM fraction was determined by the Bradford protein assay (Bio-Rad) and by OD_{280} reading.

Binding assays. The binding of purified rotavirus particles and of affinity-purified GST–fusion proteins to MA104 cells in suspension was performed by a nonradioactive binding assay essentially as described by Zárate *et al.,* (2000). Briefly, a suspension of 5×10^4 cells preincubated with the appropriate dilution of MAb 2D9 or control IgM for 1 h at 4°C was mixed either with purified virus or with recombinant proteins (previously sonicated and centrifuged for 2 min in the Eppendorf centrifuge) in MEM–1% bovine serum albumin (BSA) in a final volume of 200 μl and incubated for 1 h at 4°C with gentle mixing. The cell–virus or cell–protein complexes were washed three times with ice-cold PBS containing 0.5% BSA and then treated with 50 μl of lysis buffer (LyB) [50 mM Tris(pH 7.5), 150 mM NaCl, 0.1% Triton X-100]. In the last wash, the cells were transferred to a fresh tube. The virus and recombinant proteins present in the lysates were quantified by ELISAs. In all binding assays of either virus or recombinant proteins, a binding control with no cells was performed.

Capture ELISAs for rotavirus particles and GST–fusion viral proteins. To detect the virus particles, goat and rabbit polyclonal sera to rotavirus were used as capture (diluted 1:10,000) and detection (diluted 1:1500) antibodies, respectively. The rotavirus proteins fused to GST were captured with the goat anti-rotavirus serum and detected with a rabbit serum to GST (diluted 1:1500). In general, the ELISA was performed as follows: polystyrene 96-well plates were coated with 100 μl of capture antibody diluted in PBS, for 2 h at 37°C. Residual free protein-binding sites were blocked by incubation with 200 μl of 1% (w/v) BSA in PBS for 2 h at 37°C. Incubation with 50 μl per well of viral or protein antigen sample in lysis buffer for 1 h at 37°C was followed by incubation with 50 μl per well of the appropriate detection antibody (see above) diluted in 1% BSA in PBS. Finally, 50 μl per well of the respective alkaline phosphatase-conjugated anti-immunoglobulin serum [goat anti-rabbit IgG or goat anti-mouse IgG (diluted 1:1500), Kirkergaard and Perry] was incubated for 1 h at 37°C, and then Sigma 104 phosphatase substrate diluted in diethanolamine buffer [100 mM diethanolamine (pH 9.4), 1 mM $MgCl_2$, 5 mM sodium azide] was added. The absorbance at 405 nm was recorded with a Microplate Autoreader EL311 (Bio-Tek Instruments).

Cloning, expression, and purification of GST–fusion proteins. The cloning, expression, and purification of RRV GST–VP8 and GST–VP5 proteins have been described (Iša *et al.,* 1997; Zárate *et al.,* 2000).

Flow cytometry (FACS). MA104 cells grown to 80% confluence were washed and brought into a single-cell

suspension by incubation with 0.5 mM EDTA in PBS at 37°C and dispersed by gentle pipetting. Cells were collected by low-speed centrifugation (200 *g*) and resuspended in ice-cold MEM without serum, and the cell concentration was determined with a hemocytometer. In each experiment, 5×10^5 cells were incubated with either MAb 2D9 or IgM control antibody (20 μg/ml) for 1 h at 4°C, washed twice with 2% fetal calf serum in PBS, and then incubated with fluorescein-conjugated anti-mouse IgM antibodies (15 μg/ml; Biosourse, USA) for 1 h at 4°C. Fluorescence-activated cell sorting analysis was done using a FACScan flow cytometer and Cellquest software (Becton Dickinson) with appropriate gating parameters.

Biotinylation of cell surface proteins. A single-cell suspension of MA104 cells was prepared as described above. Cells (1×10^7) were incubated for 30 min at 4°C with water-soluble sulfo-NHS-biotin (2 mg/ml; Pierce) in PBS, with occasional gentle mixing. Unreacted sulfo-NHS-biotin was blocked by incubation with an equal volume of 10 mM glycine in PBS for 30 min at 4°C, and then the cells were washed twice with PBS and solubilized with LyB supplemented with 1 mM PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin for 10 min at 4°C. After lysis the cells were centrifuged for 10 min at 3500 rpm in the Eppendorf microfuge and the cell lysate was used for the SPIT assay (see below).

Radiolabeling of MA104 cell proteins. MA104 cells were grown in 6-well plates (Costar) to approximately 70% confluence. Cell monolayers were washed twice with methionine-free MEM and incubated for 16 h in radiolabeling medium [MEM with 1/20 the regular concentration of methionine, 10% dialyzed serum, and 100 μCi/ml Easy Tag Express-^{35}S protein labeling mix (NEN)]. Labeled cells were washed twice with PBS and solubilized with 1 ml of LyB as described above for the biotin-labeled cells.

Solid-phase immunoisolation technique. Direct SPIT was carried out essentially as described by Burns *et al.,* (1988). ELISA 96-well plates were coated with 50 μl of the antibody of interest (10 μg/ml) in PBS overnight at 4°C. The plates were washed four times with PBS and blocked with 0.5% gelatin in PBS for 1 h at 37°C, followed by addition of the radiolabeled or biotinylated cell lysates. After overnight incubation at 4°C, unbound lysate was removed and the plates were washed four times with wash buffer (PBS containing 0.01% SDS, 0.1% sodium deoxycholate, 1% Nonidet-P40). The bound proteins were solubilized by addition of gel sample buffer [1% SDS, 5% 2-mercaptoethanol, 0.5 M urea, 50 mM Tris–HCl (pH 6.8), 10% glycerol, 0.0025% phenol red] and boiling for 3 min. The samples were analyzed by SDS–PAGE and fluorography in the case of the ^{35}S-labeled proteins or by Western blot with streptavidin-peroxidase and enhanced chemiluminiscence (ECL, Amersham), in the case of the biotin-labeled proteins.

Indirect immunofluorescence. Semiconfluent MA104 cells grown in coverslips were fixed with 2% formaldehyde, 0.25% glutaraldehyde in PBS, for 15 min at room temperature, washed three times with PBS, and blocked with 1 M glycine in PBS (w/v), for 1 h at room temperature. After three further washes, the cells were incubated with 10 μg/ml of either MAb 2D9 or a control IgM (Sigma Chemical Co.) for 1 h at room temperature. Finally, following two more washes, the cells were incubated with a 1:20 dilution of a FITC-conjugated goat anti-mouse IgM (Sigma Chemical Co.) for 1 h at room temperature. The cells were washed three times and mounted on glass slides in 80% glycerol in PBS. The slides were analyzed by using a Bio-Rad MRC-600 confocal microscope and CoMOS MPL-1000 software.

Immunoelectron microscopy. Confluent MA104 cells in 25-cm^2 flasks were fixed with 2% formaldehyde, 0.25% glutaraldehyde in PBS, for 30 min at 4°C, washed three times with PBS, and blocked with 1% BSA in PBS for 15 min at room temperature. After three washes, the cells were incubated with 10 μg/ml of either MAb 2D9 or a control IgM (Sigma Chemical Co.) for 1 h at room temperature. Finally, following two washes with PBS, the cells were incubated with a 1:50 dilution of a peroxidase-conjugated goat anti-mouse IgM (Sigma Chemical Co.) for 1 h at room temperature. After three washes with PBS, the cells were incubated with the peroxidase substrate [0.5 mg/ml of DAB (Sigma Chemical Co.) in PBS with 0.1% H_2O_2], for 15 min at 37°C, and the reaction was stopped by removing the substrate and washing with ice-cold PBS. The cells were then detached from the flask with the aid of a rubber policeman, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Poly/Bed 812/DMP30 (Polysciences, Warrington, PA); 30-μm sections were obtained from selected areas of trimmed blocks, floated onto formvar-coated nickel grids, and counterstained for 5 min with a 2.5% uranyl acetate solution in 40% ethanol. Sections were examined and photographed in a Jeol JEM 1200 EXII electron microscope.

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