JOURNAL OF VIROLOGY, Feb. 1986, p. 438–445
0022-538X/86/020438-08$02.00/0
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Vol. 57, No. 2

Monoclonal Antibody That Inhibits Infection of HeLa and Rhabdomyosarcoma Cells by Selected Enteroviruses through Receptor Blockade

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Received 26 July 1985/Accepted 2 October 1985

BALB/c mice were immunized with HeLa cells, and their spleen cells were fused with myeloma cells to produce hybridomas. Initial screening of culture fluids from 800 fusion products in a cell protection assay against coxsackievirus B3 (CB3) and the CB3-RD virus variant yielded five presumptive monoclonal antibodies with three specificities: (i) protection against CB3 on HeLa, (ii) protection against CB3-RD on rhabdomyosarcoma (RD) cells, and (iii) protection against both viruses on the respective cells. Only one of the monoclonal antibodies (with dual specificity) survived two subclonings and was studied in detail. The antibody was determined to have an immunoglobulin G2a isotype and protected cells by blockade of cellular receptors, since attachment of [^{35}S]methionine-labeled CB3 was inhibited by greater than 90%. The monoclonal antibody protected HeLa cells against infection by CB1, CB3, CB5, echovirus 6, and coxsackievirus A21 and RD cells against CB1-RD, CB3-RD, and CB5-RD virus variants. The monoclonal antibody did not protect either cell type against 16 other immunotypes of picornaviruses. The monoclonal antibody produced only positive fluorescence on those cells which were protected against infection, and ^{125}I-labeled antibody confirmed the specific binding to HeLa and RD cells. The results suggest that this monoclonal antibody possesses some of the receptor specificity of the group B coxsackieviruses.

Studies of the early events in human and animal cell-virus interactions have been reviewed previously (4, 10, 15, 18, 20, 24, 25, 36). In brief, the early interactions of coxsackieviruses with receptors at the cell surface consist of attachment, penetration of the plasma membrane (endocytosis), and viral eclipse leading to uncoating of the viral genome. However, as many as 80% of attached virions are eluted or dissociated into the extracellular environment with loss of the low-molecular-weight virion polypeptide VP4 (11), making it difficult to identify the molecular events in virus uncoating (28).

The receptors for the different species of prototype picornaviruses differ in susceptibility to proteolytic enzymes (38), pH and heat inactivation (39), saturation by different picornaviruses (6, 7, 13, 23, 32), and blockade by anticellular serum (2). In addition, variant receptors in genetically dissimilar individuals may select variant viruses with altered tissue tropisms (9, 33).

In continuation of a series of studies of the membrane receptors for the group B coxsackieviruses (12, 22, 27), we now report on the isolation of monoclonal antibodies that inhibit infection of HeLa cells and human rhabdomyosarcoma (RD) cells by some of the prototype and variant coxsackieviruses through receptor blockade. This development was prompted by the recent report of Campbell and Cords (5), who showed that a monoclonal antibody preparation inhibited attachment to HeLa cells of coxsackieviruses B1, B5, and B6 without blocking attachment to some other picornaviruses. Minor and co-workers (30) have obtained monoclonal antibodies which block the three immunotypes of polioviruses from attaching to HeLa cells without preventing attachment of nine other human enteroviruses. In addition, a monoclonal antibody (5a) has recently been isolated which specifically protects HeLa cells against infection by 78 serotypes of human rhinoviruses (HRV). Thus, monoclonal antibodies promise to provide a significant insight into the nature of the cellular receptors for the different picornaviruses.

MATERIALS AND METHODS

Viruses. The prototype strains of group B coxsackieviruses B1 through B6 (CB1 through CB6) used in this study have been described previously (7). The RD cell-grown variant coxsackieviruses CB1-RD, CB3-RD, CB5-RD, and CB6-RD were derived by Reagan et al. (33) and differ from prototype group B coxsackieviruses in that the RD variant viruses are hemagglutination positive, grow on RD cells, and appear to have acquired a second site for attachment to HeLa cells. The group A coxsackieviruses A9, A13, A15, A18, and A21 were obtained originally from the University of Minnesota stock collection (13). Poliovirus types 1, 2, and 3 encephalomyocarditis virus of mice, echoviruses 1, 9, 11, and 12, and adenovirus T2 were purchased from the American Type Culture Collection (Rockville, Md.). The source of HRV2, HRV14, and HRV15 (1) and echovirus 6 (E6) was decribed previously (7). Each virus immunotype was verified by neutralization with type-specific antiserum.

Cells. HeLa cells (Mandel [M] strain [11] and [R-19 strain [1]) and L cells were grown in suspension or in monolayers as described previously (11, 21). Human RD cells were cultured in monolayers by methods given before (34). Additional cells used in these studies included monkey kidney

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lines LLC-MK-2, Vero, and CV-1 as well as the Madden-Darby canine kidney line (MDCK), the Crandell feline kidney line (CRFK), human diploid fibroblasts (MRC-5), and primary cultures of murine BALB/c kidney cells. For use in virus infectivity assays, cells were removed from the plastic substrate by a trypsin-EDTA mixture (GIBCO Laboratories), Grand Island, N.Y.), suspended in 10% fetal calf serum (FeCaS-10) plus Eagle minimal essential medium (MEM), and dispensed in 48-well plastic microtiter plates (Costar, Cambridge, Mass.), using 1.5×10^5 cells per well. The cells were ready for use after incubation at 37°C for 24 h.

Virus purification and radioactive labeling. HeLa cells were grown in suspension and washed once with phosphate-buffered saline (PBS) by centrifugation at 400 × *g* for 5 min. Cells were suspended in MEM (deficient in the amino acid used for radioactive labeling) to a concentation of 10^7 cells per ml. Virus at an input multiplicity of 50 PFU per cell was added for 30 min at room temperature. After virus attachment to cells, the suspension was diluted twofold with FeCaS-2-MEM (without labeling amino acid) and incubated in a water bath at 37°C over a magnetic stirrer for 2.75 h to allow virus inhibition of host-cell protein synthesis. The isotope (100 μCi of [^{35}S]methionine; Amersham Corp., Arlington Heights, Ill.) was added, and incubation was continued for a total of 7 h at 37°C. The cells were collected by centrifugation at 400 × *g* for 5 min, washed once in PBS, and suspended in 1 ml of RSB-Mg^{2+} (0.01 M NaCl, 0.01 M Tris, 0.015 M $MgCl_2$, pH 7.35). Virus was released from the cells after three freeze-thaw cycles, and the suspension was clarified by centrifugation at 700 × *g* for 10 min. The supernatant fluid (1 ml) received 10 μl of 0.2 M disodium EDTA and 100 μl of 10% sodium dodecyl sulfate at room temperature. The virus-containing fluid was overlaid onto a 36-ml sucrose gradient (15 to 30% [wt/wt]) containing 0.5% sodium dodecyl sulfate, 0.0005 M EDTA, 0.1 M NaCl, and 0.01 M Tris buffer (pH 7.5) and centrifuged at 24,000 rpm at 25°C for 3 h and 20 min in an SW27 rotor (Beckman Instruments, Inc., Spinco Div. Fullerton, Calif.). Fractions (1.5 ml) were collected from the bottom of the gradient and counted for radioactivity, and the two fractions containing virus were pooled, diluted 10-fold in RSB plus Mg^{2+} buffer, and centrifuged in a heavy-wall plastic tube for 15 h at 30,000 rpm and 25°C in an angle-head Ti45 rotor (Beckman Instruments, Inc.). The supernatant fluid was decanted, the virus pellet was suspended in 0.5 ml of PBS, and aliquots were frozen at −70°C until used.

Preparation of monoclonal antibodies. BALB/c mice were immunized with HeLa cells (R-19 strain) over a period of 3 months by intraperitoneal inoculation of approximately 10^7 cells emulsified in complete or incomplete Freund adjuvant at 1-month intervals. After the second immunization, the mice were bled, and each serum was examined for evidence of polyclonal antibodies by the cell protection assay. Three days after the last booster inoculation (3×10^6 cells without adjuvant given intravenously), the spleens from two mice were collected, and the lymphocytes were separated and fused with SP2/0 BALB/c nonsecretor myeloma cells as described by Hughes et al. (19). Briefly, the spleen cells were washed, pelleted, enumerated, and then combined with SP2/0 mouse myeloma cells at a ratio of 10:1. The cell pellet was then exposed to a 35% polyethylene glycol (average molecular size, 1,000 daltons; Aldrich Chemical Co., Inc., Milwaukee, Wis.)–5% dimethyl sulfoxide fusion medium. The fusion medium was added slowly with gentle stirring for 2 min. The cells were pelleted at 165 × *g* for 6 min, the fusion medium was aspirated, and the fused cells were suspended in mouse hypoxanthine-thymidine medium (formula no. 83-0157; GIBCO) supplemented with FeCaS-20. The fused cell suspension was planted at 3.0×10^5 lymphocytes per well in 20 96-well microtiter plates (Costar) containing irradiated human diploid fibroblasts (MRC-5) as feeder layers. After 24 h, the cultures were exposed to hypoxanthine-aminopterin-thymidine medium. After 10 to 14 days, the supernatant fluids from wells with approximately 40% hybridoma cell confluency were assayed by the cell protection assay.

Cell protection assay for monoclonal antibodies. The cell protection assay of Campbell and Cords (5) was modified for use. Cells were grown to about 60% confluency in 48-well flat-bottom microtiter plates. The fluid phase was aspirated, and the cells received 50 μl of antibody-containing fluid per well for 1 h at room temperature. Challenge virus (50 μl per well) containing 10^4 PFU was added for 1 h at 24°C, 0.5 ml of FeCaS-3-MEM was added, and the plates were incubated at 37°C for 24 h. The wells were examined microscopically for evidence of virus-induced cytopathic effect. The titer was expressed as the reciprocal of the highest dilution of fluid giving protection in each of the wells. Duplicate or quadruplicate assays were performed on sample fluids. An anticellular polyclonal serum prepared in rabbits immunized with HeLa cells (2) served to standardize the assay and to provide a positive control.

Virus attachment inhibition assay for monoclonal antibodies. Cells grown in suspension or removed from monolayers by EDTA were washed once and suspended in FeCaS-3-MEM. Cells (2×10^5 in 100 μl) were dispensed in replicate into 1.5-ml polypropylene Eppendorf tubes, the fluid phase was removed after centrifugation, and the cells were suspended in 50 μl of antibody-containing fluid for 1 h at 24°C. The cells were centrifuged and suspended in 20 to 40 μl of radioactively labeled virus for 90 min at 24°C. Diluent (0.5 ml of FeCaS-3-MEM) was added, and the cells were centrifuged, washed once in diluent, and solubilized in 0.5 ml of 0.1 M NaOH. The solubilized cell preparation was assayed for radioactivity by scintillation counting. All samples were assayed in duplicate. An anticellular polyclonal serum prepared in rabbits immunized with HeLa cells (2) served to standardize the assay and to provide a positive control. The titer was expressed as the reciprocal of the highest dilution of fluid which inhibited attachment of virus to cells by 50% as determined from a plot of the data.

Purification and iodination of monoclonal antibody. Fluids from subcloned hybridoma cell cultures were found to contain mouse immunoglobin G2a (IgG2a) isotype as determined by gel diffusion assays with isotype-specific antiserums. The IgG was purified on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) columns. The fluids were adjusted to pH 8.1 with 1 M NaOH and passed over the columns, which were then washed with 10-fold volumes of 0.1 M Tris buffer, pH 8. The IgG was eluted with 0.1 M glycine hydrochloride buffer (pH 3.0), and fractions were collected and assayed for protein content at an optical density of 280 nm in a Gilford spectrophotometer. The IgG was purified more than 200-fold, and the final product contained 3 to 12 mg of protein per ml as determined by the Lowry method (26).

For iodination, 0.4-ml samples of purified antibody preparation received 0.05 ml of freshly prepared chloramine-T (200 μg/ml) in deionized water and 1 mCi of $Na^{125}I$ (Amersham). The reaction proceeded for 2.5 min at 4°C and was quenched with 5 μl of 2 mg of sodium bisulfite per ml

and 10 μl of a solution containing 0.1 g of NaI per ml and 1.0% bovine serum albumin. Free iodine was removed by passing the sample through a 5-ml Sepharose G-10 column (Pharmacia) prepared in PBS. The specific activity of the iodinated preparation was 6.06×10^{-13} g/cpm or 4.04×10^{-18} mol/cpm.

RESULTS

Selection of monoclonal antibodies against cellular receptors for coxsackieviruses. CB3 and CB3-RD viruses were used for selecting monoclonal antibodies against the receptors on HeLa cells and RD cells, respectively. The CB3-RD variant of CB3 (Nancy strain) shares a common receptor on HeLa cells with the parental virus (in competition studies CB3-RD blocks CB3 attachment), but in addition CB3-RD has acquired a second site for attachment to a second receptor (CB3 does not block attachment of CB3-RD) (33). Thus, finding monoclonal antibodies to each receptor should aid in their characterization. In some experiments the fluids were assayed for capacity to prevent attachment of radioactively labeled virus. However, the protection assay was favored over the attachment-inhibition assay because of ease of performance.

Initial screening of 800 fusion products for protection of the two cell lines against the two respective viruses revealed five presumptive monoclonal antibodies with three virus-cell specificities (Table 1). However, only one of these hybridomas which secreted antibodies with dual virus-cell specificity (designated Rmc CB3-CB3RD) survived two subclonings.

Virus and cell specificity of monoclonal antibody Rmc CB3-CB3RD in the cell protection assay. The finding of a monoclonal antibody with dual specificity, i.e., protection of both HeLa cells and RD cells against CB3 and CB3-RD, respectively, was unexpected. A number of experiments were performed to determine whether the hybridoma cells were made up of a mixed cell population.

Culture fluids containing antibodies were absorbed with HeLa cells or RD cells, and the fluids were titrated for capacity to protect cells against their respective viruses. HeLa cells (3.5×10^7) grown in suspension were washed once in FeCaS-3-MEM and suspended in 1.0 ml of hybridoma cell culture fluid for 2 h at 24°C. The cells were then collected by centrifugation, and the supernatant fluid was reabsorbed with fresh cells. The fluid was titrated in the cell protection test. In a parallel experiment another sample of the same hybridoma culture fluid was absorbed by monolayers of RD cells in 75-cm^2 flasks. Three consecutive absorptions of 3 h each at 24°C were carried out, using a fresh flask of RD cells each time. Results of titrations of these fluids showed that the preabsorption titers decreased from 1:20 to 1:2 for both CB3 on HeLa cells and CB3-RD on RD cells after absorption by each cell line, respectively. Furthermore, fluids from each of 85 subclones of hybridoma cells derived from two consecutive subclonings retained the dual virus-cell specificity. It was concluded that the hybridoma culture fluid contained a monoclonal antibody with dual specificity and not a mixture of antibodies.

TABLE 1. Monoclonal antibodies with three coxsackievirus-cell specificities identified in screening of fusion products[a]

Challenge virus	Cell line	Monoclonal antibody designation
CB3	HeLa	Rmc CB3
CB3-RD	RD	Rmc CB3RD
CB3 and CB3-RD	HeLa and RD	Rmc CB3-CB3RD

[a] BALB/c mice were immunized with HeLa cells, and numerous presumptive monoclonal antibodies with each of the above specificities were found in repeated screening assays.

TABLE 2. Virus and cell specificity of the monoclonal antibody Rmc CB3-CB3RD in a cell protection assay[a]

Virus	Protection of[b]:	
	HeLa cells	RD cells
CB-1, CB3, CB5	+	NT[c]
CB2, CB4, CB6	−	NT
CB1-RD, CB3-RD, CB5-RD	−	+
CB6-RD	−	−
CA21	+	−
CA13, CA15, CA18	−	NT
CA9	NT	−
Polio virus types 1, 2, 3	−	−
E6	+	+/−
Echoviruses 1, 9, 11, 12	−	−
HRV2, HRV14, HRV15	−	−
Encephalomycarditis	−	−

[a] No protection by the monoclonal antibody was found when it was used on primary mouse kidney cells and Vero cells and challenged with CB1 through CB6 or CB1-RD through CB5-RD, respectively.
[b] +, Cell protection; −, no cell protection; +/−, partial cell protection.
[c] NT, Not testable; virus produces no cytopathic effect in control cells.

Hybridoma culture fluids obtained from cells after the two subclonings were examined for capacity to protect HeLa cells and RD cells against infection by 27 different picornaviruses (Table 2). Culture fluids were tested at dilutions of 1:2 and 1:10, with the titer for protection in the homologous systems being 1:20. Assays for protection of the cells against the group B coxsackieviruses revealed some unexpected results. First, only CB1, CB3, and CB5 were inhibited in HeLa cells, whereas CB2, CB4, and CB6 were uninhibited, even though in virus competition studies all six group B viruses compete for the same receptor. In this regard it is important to note that the odd-numbered CB viruses attach to HeLa cells much more rapidly than the even-numbered CB viruses (8). Second, three of the four CB-RD variants were inhibited on RD cells (CB6-RD was not inhibited), even though in virus competition studies (33) CB3-RD was not blocked in attachment to HeLa cells by parental CB3. Third, only the human cell lines HeLa and RD were protected, whereas primary mouse kidney cells and Vero monkey kidney cells were not protected. Additional findings revealed that E6 was inhibited, but not four additional immunotypes of echoviruses; coxsackievirus A21 was inhibited, but not four other group A immunotypes; HRV14 was not inhibited, even though in virus competition studies coxsackievirus A21 and HRV14 share the same receptor (23). On the other hand, the monoclonal antibody did not protect cells against the three immunotypes of poliovirus, three immunotypes of HRV, or encephalomyocarditis virus.

Inhibition of virus infection by the monoclonal antibody through receptor blockade. Many experiments were performed in which the culture fluids and purified antibody preparations from the hybridoma cells were titrated for capacity to inhibit attachment of labeled virus to cells (see Materials and Methods). The 50% endpoint titer of culture fluids generally ranged from 1:20 to 1:60, whereas for purified antibody a titer of 2,920 for a sample containing 3.3 mg of IgG protein per ml was found (Fig. 1). Saturation of cellular receptors was achieved at a dilution of 1:400, which

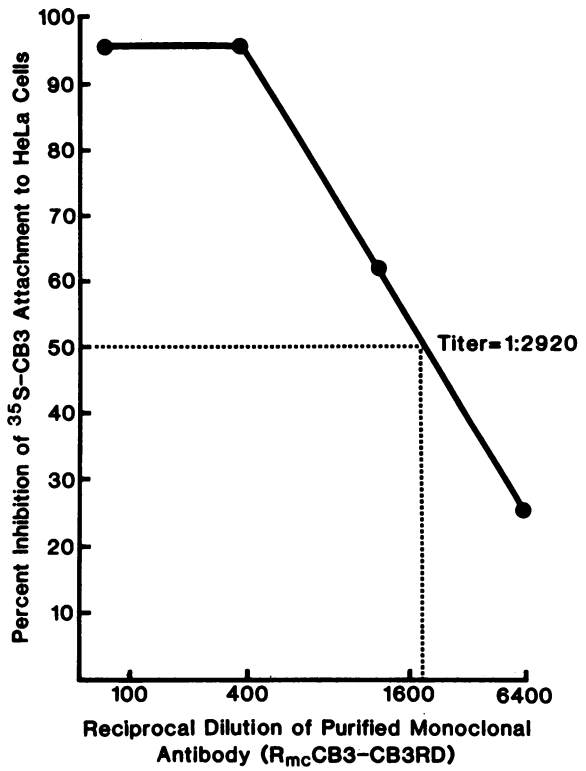

FIG. 1. Inhibition of binding of [^{35}S]methionine-labeled CB3 to HeLa-M cells pretreated with purified antireceptor monoclonal antibody (Rmc CB3-CB3RD).

reflected 95% inhibition of attachment of labeled virus. Thus, approximately 0.41 μg of IgG protein saturated the 2 × 10^5 cells in the test (0.0021 ng per cell). In repeated experiments this value ranged from 0.39 to 0.58 μg of IgG protein.

To determine whether the monoclonal antibody would block the attachment of [^{35}S]methionine-labeled CB3, CB3-RD, and E6 cells to an assortment of cells representative of several animal species, we performed experiments using a 1:200 dilution of purified antibody to treat 2 × 10^5 cells in the standard inhibition assay. The results (Table 3) were unexpected in that the monoclonal antibody only blocked virus attachment to cells of human origin (HeLa and RD) and not to monkey or canine kidney cells.

TABLE 3. Cell specificity of the monoclonal antibody

Cell type	% Inhibition of virus attachment[a]	
	CB3	CB3-RD
HeLa	91	65
RD	—[b]	100
MK-2	<10	<10
Vero	13	<10
CV-1	<10	26
MDCK	10	<10

[a] E6 attached only to HeLa cells (42%). This attachment was totally inhibited by the monoclonal antibody. There was no significant attachment of E6 to any of the other cells.

[b] —, No attachment of virus to control cells; also none of the three viruses attached to mouse L cells or to feline CRFK cells. The amounts of [^{35}S]methionine-labeled virus used were: CB3, 6,400 cpm; CB3-RD, 4,600 cpm; E6, 2,900 cpm.

Evidence that CB3 does not share a receptor with E6. Previous studies from our laboratory have shown that CB3 and E6 do not compete for the same receptor on HeLa cells (7). However, the finding that a monoclonal antibody blocked attachment of both of these viruses and of CB3-RD suggested a need to perform additional virus competition experiments with radioactively labeled viruses.

HeLa cells grown in suspension were washed once in FeCaS-3-MEM and resuspended to 3 × 10^6 cells per ml, and 0.1 ml was dispensed into 1.5-ml polypropylene tubes (Eppendorf). The cells were deposited by centrifugation (Eppendorf centrifuge), the medium was withdrawn, and the cells were suspended in 50 μl of unlabeled CB3 (3 × 10^8 PFU) to give a virus multiplicity of 1,000 PFU per cell. This amount of virus is known to saturate the CB3 receptors (7). An equal number of tubes received medium without virus. The suspensions were incubated for 1 h at 6°C, and the cells were washed once with cold FeCaS-3-MEM (0.5 ml per tube) and suspended in [^{35}S]methionine-labeled CB3, CB3-RD, or E6 (40 μl per tube). The attachment of each virus was assayed in quadruplicate, using a 60-min attachment time at 20°C. The cells were washed twice with FeCaS-3-MEM (0.5 ml each time) and solubilized in 0.5 ml of 0.1 M NaOH, and the amount of cell-associated radioactivity was determined by scintillation counting. Tubes without cells received equivalent amounts of labeled viruses to serve as a control of nonspecific binding of label to the walls of the tubes.

The results (Table 4) confirmed previous observations that CB3 and E6 do not compete for the same receptor. In addition, no competition between CB3 and CB3-RD was found, as shown previously by infectivity assays (33).

Use of iodinated monoclonal antibody to determine the number of antibody-binding sites on cells. Early studies revealed that high-titered preparations of CB3 would saturate the receptors on HeLa cells (7) at a value of approximately 300 to 400 PFU per cell or about 10^5 virions per cell (based on 300 virions per PFU). In addition, the results shown in Fig. 1 revealed the sites to be saturable. Therefore, experiments were performed to determine the number of antibody-binding sites on HeLa cells by use of iodinated monoclonal antibody.

A preparation of purified Rmc CB3-CB3RD was iodinated as described in Materials and Methods. Serial twofold dilutions of the antibody preparation were made in FeCaS-3-MEM, and 50-μl samples were added to HeLa cells or L cells (2 × 10^5 cells per tube) in duplicate for binding (1 h, 23°C). The cells received 0.5 ml of FeCaS-3-MEM, and they were collected by centrifugation, washed once in diluent, and counted in a gamma counter for cell-associated radioactivity. Tubes without cells were included to monitor the binding of label to the walls of the tubes (less than 1%). The results of a representative experiment (Fig. 2) show that the receptors for binding labeled antibody were saturated at a

TABLE 4. Absence of competition between CB3 and CB3-RD or E6 for receptors on HeLa cells

[^{35}S]methionine-labeled virus[a]	Amt of virus attached (%) to:		% Inhibition
	Normal cells	CB3-treated cells	
CB3	36	5	86
CB3-RD	82	81	1
E6	36	37	0

[a] Counts per minute added: CB3, 5,150; CB3-RD, 5,420; E6, 3,710.

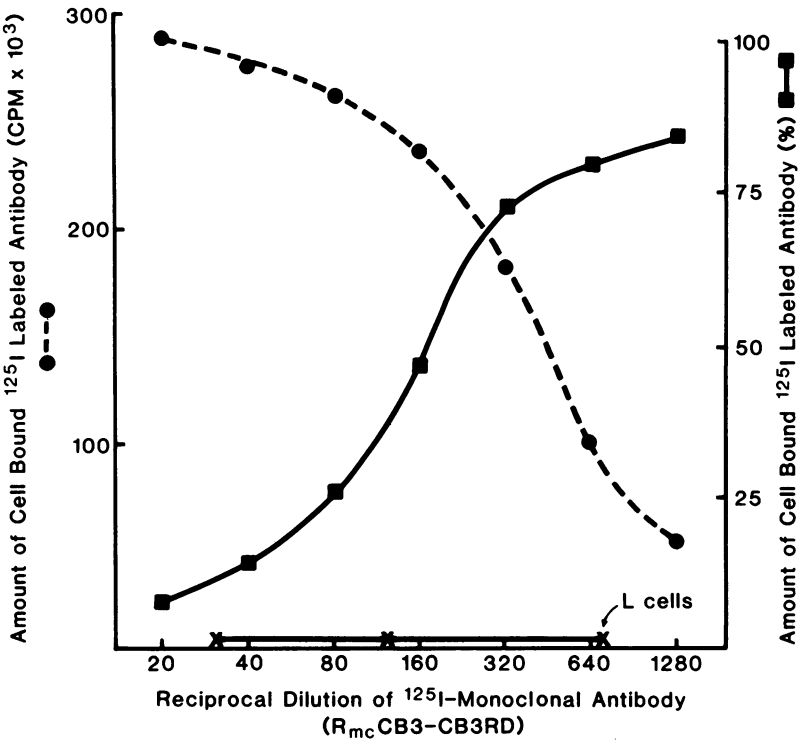

FIG. 2. Adsorption of ^{125}I-labeled monoclonal antibody Rmc CB3-CB3RD to HeLa-M and L cells.

dilution of 1:20 (lowest dilution tested) and that as the antibody preparation was diluted, an increasing percentage of label was bound. The finding that 80% of the label was bound to HeLa cells suggested that the iodination procedure did not destroy the binding capacity of the monoclonal antibody. A Scatchard plot of the data revealed that there were 3.5×10^6 binding sites per HeLa cell. It is also evident that less than 0.02% of the label bound to L cells.

To confirm the specificity of the iodinated monoclonal antibody for binding to HeLa and RD cells, a competition experiment was performed with differing dilutions of unlabeled, purified monoclonal antibody and a constant amount of labeled antibody. The results (not shown) revealed that the unlabeled antibody blocked attachment of the iodinated antibody to both HeLa and RD cells. In addition, the RD cells bound only 6% of the labeled antibody compared with that which bound to HeLa cells, suggesting fewer receptor sites for the monoclonal antibody on RD cells.

Evidence for a cell surface antigen for the monoclonal antibody as detected by immunofluorescence. To further demonstrate that the Rmc CB3-CB3RD antibody reacted with an antigen located at the surface of the plasma membrane of HeLa cells and RD cells and to confirm the cell species specificity of this antibody, a series of experiments were performed using an indirect immunofluorescence assay. A variety of cells of different origins including HeLa-M, RD, Vero, CV-1, BHK21, MDCK, 1° and 2° cultures of monkey kidney, Swiss 3T3, primary and secondary cultures of BALB/c kidney cells, and L cells were grown in eight-well slide cultures (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) to approximately 40 to 80% confluency. The cells were washed with MEM and overlaid with a 1:100 dilution of purified monoclonal antibody for 1 h at room temperature. The cells were washed twice with PBS, and a 1:80 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Tago, Inc.) was added for 1 h at room temperature. The cells were washed again with two changes of PBS and examined for cell-associated fluorescence with a Zeiss microscope equipped with an epifluorescence condenser, a halogen illuminator, a 63X planapochromatic objective, and appropriate filters. Control preparations included untreated cells and cells treated with a mouse monoclonal antibody with specificity for poliovirus capsid antigen (16).

The results of these experiments revealed that only HeLa and RD cells showed positive immunofluorescence in both unfixed preparations and in cells fixed in ethanol at −20°C. A representative photomicrograph of an unfixed HeLa cell preparation (Fig. 3) reveals an extensive and rather uniform distribution of antigen on the cell surface. The RD cells showed a positive but very low level of fluorescence, which was consistent with the results of binding studies with the iodinated antibody preparation. Absence of positive immunofluorescence on all of the other cells tested was in agreement with the results of cell protection tests. It was concluded that the monoclonal antibody reacted only with cells of human origin and not with cells of other species tested, even though they possess receptors and are susceptible to CB3 infection, e.g., monkey kidney cells and primary mouse kidney cells.

Attempts to obtain a CB3 variant which would be resistant to the protective effect of the Rmc CB3-CB3RD antibody. It has been found that polioviruses which are resistant to virus-neutralizing monoclonal antibodies can be selected after passage of virus in the presence of these antibodies (16). Experiments were performed to determine whether virus variants could be obtained for CB3 which would be resistant to the cell-protective effect of the receptor monoclonal antibody.

HeLa-M cells in six-well cluster plates (Costar) were

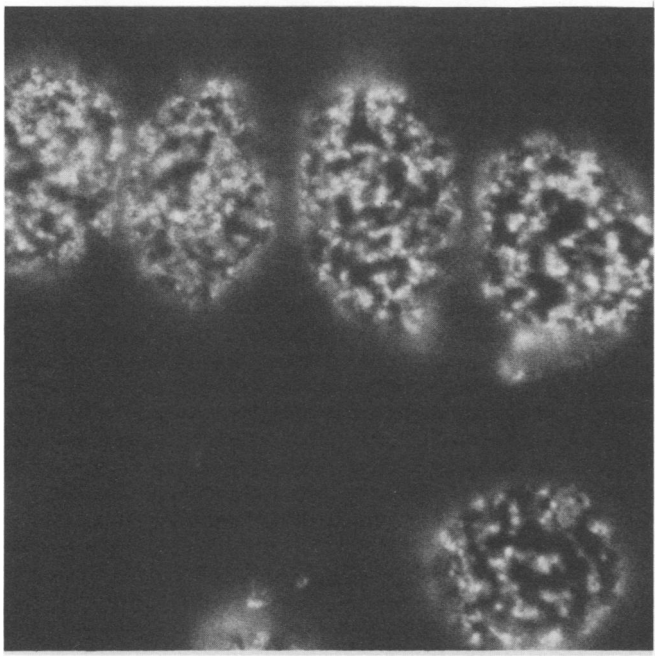

FIG. 3. Photomicrograph of unfixed HeLa-M cells after reaction with the purified monoclonal antibody Rmc CB3-CB3RD and a fluorescein-labeled goat anti-mouse IgG.

treated with a 1:50 dilution of purified monoclonal antibody for 1 h at 24°C and inoculated with CB3 to give approximately 100 plaques in untreated plates. Virus was allowed to attach to cells for 1 h at room temperature, and the plates were overlaid with FeCaS-3-MEM–1% Bacto-Agar (Difco Laboratories, Detroit, Mich.) containing antibody diluted 1:200. After 3 days of incubation at 37°C, the plates were inspected for the presence of plaques. The virus control plates had approximately 100 plaques each, whereas the monoclonal antibody-treated plates showed only 5 to 6 plaques each. Representative plaques were picked and titrated for passage under similar conditions as described above. After eight serial passages of CB3 virus plaques on HeLa cells treated with monoclonal antibody, a number of plaques comparable to that of the first passage was still recovered. Thus, it was concluded that the virus population did not produce variants which became resistant to the cell-protective effects of the monoclonal antibody. Presumably the 3 to 6% of plaques which developed in the presence of anticellular antibody were not virus variants.

Capacity of the monoclonal antibody to protect mice against death by CB3. Minor and co-workers (30) found a protective effect against poliovirus after treatment of monkeys with a monoclonal antibody against the poliovirus receptor. Similarly, we attempted to determine whether the Rmc CB3-CBRD antibody will protect young adult BALB/c mice against lethal challenge by CB3 (Woodruff strain).

Groups of 12 BALB/c mice were each given 0.1 ml amounts of purified monoclonal antibody (100 μg of antibody protein) by the intraperitoneal route daily. The first inoculation was started 4 days before the administration (intraperitoneally) of 1.0-ml amounts of CB3 per mouse. Groups of mice were challenged with 10^3 and 10^4 PFU of CB3, which were determined previously to provide approximately 1.0 and 10 50% lethal doses of virus, respectively. Equal numbers of mice received daily inoculations of PBS instead of monoclonal antibody to serve as controls for virus lethality. The animals were examined daily for 21 days postinfection for signs of infection and death. The results (Table 5) reveal that the monoclonal antibody provided some protection of the mice against death by a minimal lethal dose of CB3. The mice receiving the monoclonal antibody and virus became noticeably ill and emaciated but survived better than did the virus-control animals. These results suggest that at least some mouse-specific cells which bear receptors for CB3 are also capable of binding the antibody.

DISCUSSION

To date several monoclonal antibodies have been obtained which have specificity for binding to cellular receptor sites for different picornaviruses. These monoclonal antibodies include the one for receptors on HeLa cells for CB1, CB5, and CB6 obtained by Campbell and Cords (5), one for the receptor on HeLa, RD, and monkey kidney cells for the three poliovirus immunotypes obtained by Minor et al. (30), one for receptors on HeLa cells for the major group of HRVs found by Colonno et al. (5a), and the one reported herein, which has an extended receptor specificity to completely block receptors for CB1, CB3, CB5, E6, and coxsackievirus A21 on HeLa cells and CB1-RD, CB3-RD, and CB5-RD on RD cells. In general these monoclonal antibodies help to confirm the existence of separate receptor families for the different species of picornaviruses, which first were recognized by virus competition experiments (1, 3, 6, 7, 14, 23, 29, 32, 34).

The finding that the monoclonal antibody Rmc CB3-CB3RD blocked receptors for some picornaviruses outside of the specific receptor family (i.e., E6 and coxsackievirus A21) was unexpected (6, 8). In addition, the monoclonal antibody blocked attachment to RD cells of CB1-RD, CB3-RD, and CB5-RD variant viruses, which presumably use the same receptor site on HeLa and RD cells as well as a second site on HeLa cells. The latter site is shared by the CB3 parental virus (33). Thus, the Rmc CB3-CB3RD antibody blocks the HeLa-specific receptor but blocks the RD/HeLa receptor only on RD cells. In this regard, a sample of the monoclonal antibody of Campbell and Cords (5) was found to block attachment to HeLa cells of CB3, but it did not block attachment of CB3-RD virus to RD cells. Thus, this IgM-class monoclonal antibody showed one of the antibody specificities for cellular receptors that we observed in our screening assays (Table 1) and helped confirm the dual receptor specificity found for Rmc CB3-CB3RD. One may explain the results observed for the Rmc CB3-CB3RD antibody by postulating that the antibody-combining site exists on a subunit which is variously associated with the cellular receptor complexes for different enteroviruses. Alternatively, the antibody site may be variously expressed on the polypeptide chain which bears the receptor. Perhaps the

TABLE 5. Protection of BALB/c mice by the monoclonal antibody against lethal challenge by CB3

Amt of virus inoculated (PFU)	No. of animals dead[a]	
	Virus + placebo	Virus + antibody
10^3	8	0
10^4	12	12

[a] 12 mice in each group received PBS or monoclonal antibody as described in the text. Values represent dead animals at 21 days postinfection. Additional control mice were inoculated with antibody daily for the duration of the experiment and all remained healthy.

capacity of the antibody to block the receptor site may occur by induced conformational effect or by steric hindrance.

Preliminary studies have shown that the Rmc CB3-CB3RD antibody binds to a 50-kilodalton cellular protein as detected by immunoblotting. This protein is similar in size to that of a protein designated Rp-a, which recently was isolated and shown to be a cellular receptor protein with specificity for binding group B coxsackieviruses (27). The cellular receptor site for the group B coxsackieviruses, however, has been found to have an approximate molecular size of 275 kilodaltons (22). Additional studies are needed to determine the relationship between Rp-a and the 50-kilodalton protein and their assignment to the larger cellular receptor complex for the group B coxsackieviruses.

The group B coxsackieviruses replicate in mice and usually cause a fatal disease. Consequently, certain mouse cells must express receptors for these viruses. The mice should recognize these endogenous receptor proteins as self antigens and, barring the development of autoimmune antibodies, they are not likely to respond to identical epitopes presented on a foreign cell protein. Thus, the monoclonal antibody produced against the HeLa cell receptor for the group B coxsackieviruses (5) and the one described herein may be directed only to those epitopes on the receptor that are proximal to the functional site or even on an adjacent protein. Presumably, this situation differs from that posed for an antibody response against receptors for polioviruses (30) and HRV14 (5a) which do not replicate in mice due to lack of cellular receptors (17, 37). For these latter two virus-receptor systems, murine monoclonal antibodies have been recovered that appear to block directly the respective functional receptor sites, since their specificity coincides with the results of virus competition for specific cellular receptors. Nevertheless, it is likely that more monoclonal antibodies of differing specificities will be needed to help define the structure of the different cellular receptors for the picornaviruses and their variant viruses. In addition, these antibodies should be helpful in determining the relationship between receptors occurring on different species of animals (31) and on different tissues undergoing differentiation (34). Finally, such reagents will be useful to help explain variation in the production of disease among individuals, since receptors are important determinants of virus tropism in pathogenesis (10, 35).

The finding of some protective effect against challenge by CB3, provided by the administration of monoclonal antibodies in young adult mice, suggests that monoclonal antibodies may be useful as viral prophylactic reagents. Alternatively, the monoclonal antibody-receptor system may be used for developing a drug antagonist. Additional animal studies of these systems seem warranted.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous gift of monoclonal antibody by Carl Cords. R.L.C. would like to thank Edward M. Scolnick for the opportunity to spend a 6-month sabbatical leave from Hahnemann University to perform research at the Merck Sharp & Dohme Research Laboratories and for many stimulating discussions.

A portion of this research was supported by Public Health Service grant AI-03771 from the National Institute of Allergy and Infectious Diseases.

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