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A Monoclonal Antibody That Blocks Poliovirus Attachment Recognizes the Lymphocyte Homing Receptor CD44

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A monoclonal antibody, AF3, was previously shown to specifically inhibit poliovirus binding to HeLa cells and to detect a 100-kDa glycoprotein only in cell lines and tissues permissive for poliovirus infection. These results suggested that the 100-kDa protein may be involved in the pathogenesis of poliomyelitis and the cellular function of the poliovirus receptor site. To study further the role of the 100-kDa protein in poliovirus attachment, immunoaffinity purification, amino acid sequencing, and cDNA cloning were undertaken. The results demonstrate that antibody AF3 reacts with the lymphocyte homing receptor CD44, a multifunctional cell surface glycoprotein involved in the homing of circulating lymphocytes to lymph nodes and the modulation of lymphocyte adhesion and activation. Antibody AF3 reacts with a subset of CD44 molecules ($_{AF3}$CD44H), which appears to be a small fraction of the heterogeneously glycosylated CD44 molecules expressed on hematopoietic and nonhematopoietic cells. Anti-CD44 monoclonal antibodies, previously reported to induce CD44-mediated modulation of lymphocyte activation and adhesion, compete with ^{125}I-AF3 in binding assays, demonstrating functional overlap among the epitopes. The anti-CD44 monoclonal antibody A3D8, which binds to a greater molecular weight range of CD44 than does AF3, inhibits poliovirus binding to a similar degree. CD44 does not act as a poliovirus receptor, since CD44-expressing mouse L-cell transformants did not bind poliovirus. The poliovirus receptor and $_{AF3}$CD44H may be noncovalently associated, or they may interact through the cytoskeleton or signal transduction pathways.

Poliovirus, the causative agent of poliomyelitis, is a member of the *Picornaviridae*, a family of single-stranded RNA viruses that causes a variety of human diseases (9). Infection of susceptible cells begins when the viral capsid, which consists of 60 copies of each of four proteins arranged in icosahedral symmetry, binds to a receptor site on the cell surface. The poliovirus receptor (PVR) has been identified as a member of the immunoglobulin superfamily of proteins (29).

Several monoclonal antibodies that block poliovirus binding and infection have been isolated that are directed against the surface of susceptible cells. Antibodies 280, D171, and 711C specifically block poliovirus infection and are directed against the first immunoglobulin-like domain of the PVR (30, 31, 36). These antibodies differ from monoclonal antibody AF3, which preferentially blocks the binding and cytopathic effect of poliovirus type 2 and detects a 100-kDa protein only in tissues and cell lines permissive to poliovirus infection (39). In an immunohistochemical survey of the human brain stem, antibody AF3 binding precisely correlated with those areas that are damaged by poliomyelitis of the brain stem, including the periaqueductal motor nuclei (3, 39), suggesting that the 100-kDa protein may be involved in the pathogenesis of poliomyelitis. In contrast, PVR mRNA and protein are expressed in tissues that poliovirus does not normally bind and infect (8, 29).

Here we report that the 100-kDa protein recognized by antibody AF3 is the lymphocyte homing receptor CD44. CD44, a member of the cartilage link family of proteins (43), is a heterogeneously glycosylated, multifunctional family of molecules identified initially with monoclonal antibodies (4, 5, 12, 13, 17, 21–24, 45, 46). The CD44 family of proteins comprises three size classes: the major species found in hematopoietic (CD44H) and nonhematopoietic cells have molecular sizes ranging from 80 to 110 kDa (5, 7, 16, 20, 23, 33, 42–44); a species primarily associated with epithelial cells (CD44E) has molecular sizes ranging from 130 to 160 kDa (7, 33, 35, 43, 44); and a minor species associated with a variety of cell types is modified by chondroitin sulfate, resulting in molecular sizes ranging from 180 to 215 kDa (23, 33, 43, 44). The 100-kDa protein is the hematopoietic isoform CD44H, which is considered to play major roles in lymph node homing for circulating lymphocytes, binding of hyaluronic acid, lymphocyte activation, and homotypic and heterotypic cell adhesion (1, 2, 6, 15, 19, 22, 24, 25, 42). CD44H has a predicted extracellular domain of 248 amino acids, a 21-amino-acid transmembrane domain, and a 72-amino-acid cytoplasmic domain (43). The 100-kDa variant of CD44H has been demonstrated to be involved in poliovirus attachment, and its tissue distribution correlates with the tropism of poliovirus, yet it does not act as a receptor for poliovirus. CD44H may be either a component of the virus receptor site or it may interact with the PVR through the cytoskeleton or signal transduction pathways.

MATERIALS AND METHODS

Purification of antibody AF3 and linkage to agarose support. Purification was carried out by using the MAPS II Protein-A immunoglobulin purification kit (Bio-Rad) with modifications. Two columns were used, one containing 100 ml of Sepharose CL-4B (Sigma; CL-4B-200) for separating the products of ascites and the other containing 1.5 ml of protein A-agarose. The Sepharose and protein A columns were equilibrated with MAPS II binding buffer. After centrifugation at

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10,000 × *g* for 10 min at 4°C, 3 ml of AF3 ascites was mixed with 0.2 ml of 0.2% dextran blue–0.2 ml of 0.2% pyronin Y and added to the Sepharose column. After the dextran had passed out of the column, the Sepharose column efflux was passed through the protein A column. When the pyronin Y dye front reached the bottom of the Sepharose column, the protein A column was disconnected and washed with binding buffer. Antibody was eluted from the column as outlined in the MAPS II instruction booklet. The antibody was dialyzed overnight against 500 volumes of 0.2 M potassium phosphate, pH 7.4, and then it was concentration dialyzed. Coomassie brilliant blue stain of samples subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated high purity.

Three milliliters of purified AF3 containing 1.9 mg of immunoglobulin per ml was linked to Affi-Gel 10 (Bio-Rad). Antibody solution was diluted to 0.1 M potassium phosphate, pH 7.4. Affi-Gel 10 beads were washed three times in ice-cold distilled water and then twice in 0.1 M potassium phosphate. The packed beads were washed once more in buffer, and then antibody solution (ca. 6 mg) was added. The slurry was rocked on ice for 2.5 h, after which 0.1 ml of 1 M glycine ethyl ester was added. The preparation was rocked on ice for an additional hour, after which the beads were resuspended and washed in phosphate-buffered saline (PBS)–0.1% sodium azide and stored as a slurry with PBS-sodium azide at 4°C. The gamma fraction of goat anti-human serum albumin (ca. 7 mg) from TAGO was also linked to 2 ml of Affi-Gel 10 by the same protocol.

Immunoaffinity isolation of the 100-kDa protein. For initial immunoaffinity purification, human peripheral mononuclear cells were solubilized for 30 min on ice in TPI (25 mM Tris [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 5 mM EDTA–20 μg of pepstatin per ml, and 2 μg of leupeptin per ml) containing 0.5% deoxycholate (DOC). The solubilized cells were centrifuged at 100,000 × *g*, and immunoaffinity isolation was carried out on the supernatant as described for large-scale immunoaffinity purification. For large-scale immunoaffinity isolation, surgical specimens of human spleen (140 to 190 g) were used. The specimens were stored frozen at −70°C, after a postoperative interval of less than 4 h. The tissue was thawed and minced in a Waring blender with 70 ml of TPI. Additional proteinase inhibitors were added to account for the volume of the spleen when thawed. The minced spleen was then centrifuged at 100,000 × *g* to pack the tissue. The supernatant was removed, and the crude pellet was solubilized in 100 ml of TPI containing 0.5% DOC. The solubilization proceeded for 80 min on ice, after which the preparation was centrifuged at 140,000 × *g* for 1.5 h at 4°C. Supernatant was removed and returned to the centrifuge for an additional 30 min at 140,000 × *g* at 10 to 15°C. The supernatant was carefully removed and was ready to be loaded onto the chromatography system.

The immunoaffinity chromatography system used to purify the 100-kDa protein consisted of a 2-ml precolumn containing ca. 7 mg of anti-human serum albumin immunoglobulin connected in a series with a 0.6-ml antibody AF3 column containing ca. 6 mg of immunoglobulin. The system was equilibrated with 100 ml of 25 mM Tris HCl–5 mM EDTA–0.5% DOC, pH 7.6 (TED). The 0.5% DOC, 140,000 × *g* spleen supernatant was loaded onto the system at 2 ml/h at 4°C and run for 45 h. The system was emptied of sample in the forward direction with TED containing 0.55% DOC at a flow rate of 2 ml/h. The AF3 column was then isolated from the rest of the system and washed in the reverse direction with 2,000 bed volumes of TED–0.55% DOC. The AF3 column was then equilibrated with TED containing 0.11% DOC. The bound material was eluted with 50 mM diethylamine–0.11% DOC, pH 11.5. Fractions were collected every 0.6 ml. Samples (30 μl) of each fraction were assayed by a dot-blot immunoassay, using antibody AF3 as probe and a goat anti-mouse-alkaline phosphatase conjugate (Promega Biotec). Fractions containing high levels of AF3 immunoreactivity were pooled directly onto dialysis membrane (Spectrum). The pooled material was dialyzed against 6 liters of high-pressure liquid chromatography (HPLC) grade H_2O (Aldrich) once overnight at 4°C. The dialysate was lyophilized. Silver staining of the isolated material was performed on SDS-polyacrylamide gels to assess purity and amount of material isolated.

Generation of the 65-kDa CNBr fragment for partial internal amino acid sequencing. The protein sample was resuspended in water containing 0.1% SDS, and formic acid (100%) was added to make the sample 70% formic acid. Then, 70% formic acid containing 70 μg of CNBr per ml was added to achieve 10 μl of CNBr stock solution per 10 μg of protein. The preparation was incubated in the dark for 24 h at room temperature (RT). The formic acid and CNBr were removed from the sample first by drying under nitrogen gas and then by Speed Vac overnight. Silver stain analysis was used to assay for completion of the CNBr cleavage. The sample was then subjected to SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) by a modification of the method described in reference 27.

Immediately after subjecting the sample to SDS-PAGE, the gel was soaked in electroblot transfer buffer which consists of 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0 (CAPS buffer). The PVDF membrane was rinsed with 100% methanol and then soaked in CAPS transfer buffer. The protein was electroblotted, in CAPS buffer, onto the PVDF membrane by using a mini-Transblot cell (Bio-Rad) for 50 min at 0.45 A with ice-pack cooling. The PVDF membrane was washed in H_2O for 5 min and stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol for 5 min. The sample was destained in 50% methanol–10% acetic acid for 5 to 10 min, rinsed in HPLC grade water (Aldrich) for 5 to 10 min, air dried, and stored at −20°C. The 65-kDa fragment, which on PVDF was a band ca. 1 mm wide, was cut out of the PVDF membrane and microsequenced by the Harvard University Microchemistry Laboratory.

Molecular cloning of CD44H cDNA. CD44H cDNA was generated by PCR. Primer design was based on GenBank sequences for CD44. The primers used were CD44cncf (5′CCAGCCTCTGCCAGGTTC3′), which corresponds to a site 114 bp 5′ from the start codon, and CD44rev (5′GTACG GAATTCTTACACCCCAATCTTCA3′), which corresponds to the end of the coding region of CD44 encoding the 72-amino-acid cytoplasmic domain and has an *Eco*RI site built into its 5′ end. This pair of primers would amplify cDNA encoding CD44H and most other isoforms of CD44. The template cDNA was from an activated T-cell library in the plasmid pcD (32), which was kindly provided by Gordon Freeman (Dana-Farber Cancer Institute). Two size-fractionated libraries were used: no. 122 corresponded to 1- to 2-kb cDNA, and no. 123 corresponded to 2- to 4-kb cDNA. The polymerase used was from *Pyrococcus furiosus* (Stratagene). A 1,200-bp product was generated from library no. 122 which by automated chain termination sequencing and restriction mapping was identified as CD44H. The *Pfu* PCR product was treated with Sequenase enzyme and ligated into Bluescript at an *Eco*RV site in the polylinker. Clones were picked by color selection and identified as full-length clones by restriction digestion and automated chain termination DNA sequencing.

CD44H inserts were isolated by digestion with *Hin*dIII and *Not*I and ligated into pcDNA1/NEO (Invitrogen). Clones were selected by ampicillin and tetracycline resistance on MC1061/p3 bacteria and tested for orientation by restriction digestion. Clones 13, 21, and 33 contained full-length inserts in the correct orientation.

Generation of CD44H-transformed L-cell lines. Stable L-cell transformants were generated by the calcium phosphate-DNA coprecipitation method (38). Ten million L cells were treated with coprecipitate containing 10 μg of DNA linearized with *Sac*II. Cell lines were tested for expression of the AF3 epitope first by immunofluorescence to establish the percentage of expressing cells in the initial transformant line. Between 50 and 70% of the cells transformed with CD44H cDNA from clones 13, 21, and 33 expressed protein which reacted with antibody AF3 but not with the anti-PVR antibody 711C or an irrelevant antibody. From all three lines, subclones were established and isolated by horseradish peroxidase-enzyme-linked immunosorbent assay, using AF3 as probe. The clones were expanded and tested by immunofluorescence again with AF3 to ensure that 100% of the cells expressed the AF3 epitope.

Immunohistochemical assays. Expression of CD44H or the PVR protein was assayed immunohistochemically by using confluent 6-well plates containing the stable transformant L-cell lines 33.2 (CD44H-pcDNA1/NEO), 13.1 (CD44H-pcDNA1/NEO), 20B (PVR-pSVL), PVR.neo.1 (PVR-pcDNA1/NEO), normal L cells, and HeLa cells. The antibodies tested were AF3, two anti-CD44 monoclonal antibodies, P3H9 and A3D8 (Sigma), and the anti-PVR monoclonal antibody 711C. Confluent monolayers were incubated with each of the monclonal antibodies for 45 min at 37°C and 5% CO_2. The monolayers were washed twice with tissue culture medium and incubated for 45 min at 37°C in 5% CO_2 with goat-anti-mouse fluorescein isothiocyanate or rhodamine (TAGO) diluted 1:60 in culture medium. The monolayers were washed twice for 15 min with tissue culture medium at RT and then washed once for 30 s with PBS. The monolayers were immediately sealed with Fluoromount-G (Sigma) and coverslips.

Radiolabeling of antibody AF3 and PV2w2. Poliovirus type 2w2 (PV2w2) was obtained from the American Type Culture Collection and radiolabeled as described previously (39). Specific activities of 7×10^{-3} cpm/PFU were obtained. AF3 was radiolabeled by the chloramine T method as previously described (10, 18, 41). Specific activities of 2×10^6 cpm/μg were obtained.

^{125}I-AF3 antibody competition assays. To test antibodies for the ability to inhibit ^{125}I-AF3 binding, confluent monolayers of HeLa S3 cells in 6-well plates were washed once with medium in order to remove loose cells. The anti-CD44 antibodies tested were A3D8 and IM7 (PharMingen). Antibody 5E2B4 is an irrelevant antibody which binds to an intracellular antigen. Three hundred microliters of medium was added to each well with 20 μg of each antibody to be tested. The monolayers were incubated at 37°C with 5% CO_2 for 40 min. ^{125}I-AF3 was added at 10^5 cpm per well and incubated for 1 h at 37°C with 5% CO_2. The monolayers were then washed twice with medium for 10 min each. Two hundred microliters of 1 M NaOH was added, and the plates were incubated overnight at RT. The samples were then assayed for radioactivity.

Assays of antibody-induced inhibition of radiolabeled poliovirus binding. To test antibodies for the ability to inhibit the binding of ^{35}S-PV2w2, confluent HeLa S3 monolayers in 6-well plates were washed once with medium to remove loose cells. The negative control antibodies were 5E2B4 and TS1/22 (anti-LFA-1; ATCC no. HB202). Antibodies were added at 10 μg in 300 μl of medium per well and incubated for 2 h at 37°C in 5% CO_2. ^{35}S-PV2w2 was added at 1.5×10^4 cpm per well in 50 μl of medium and incubated for an additional 30 min at 37°C in 5% CO_2. The monolayers were washed twice with prewarmed medium for 5 min each. Then 200 μl of 1 M NaOH was added, and the plates were incubated overnight at RT. Two hundred microliters of 1 M HCl and 6 ml of Aquasol-2 (New England Nuclear) were added, and the samples were assayed for radioactivity. The binding kinetics were such that less than 15% of radiolabeled virus was allowed to bind to the HeLa cells. In this way differences in the initial association rates, after pretreatment of the monolayers with antibody, could be determined.

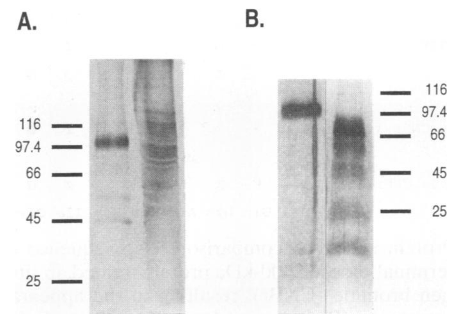

FIG. 1. (A) Silver stain analysis of protein from immunoaffinity purification of the 100-kDa protein, using human peripheral mononuclear cells. Left lane, immunoaffinity-purified protein; right lane, whole DOC 100,000 × *g* supernatant before immunoaffinity chromatography. (B) Silver stain analysis of preparatively purified 100-kDa protein from human spleen tissue, before and after cyanogen bromide treatment. Left lane, 100-kDa protein untreated; right lane, fragments generated by 24-h cyanogen bromide treatment in 70% formamide. Panel A is 10% and panel B is 12.5% polyacrylamide.

Infectivity assays. PV2w2 stock lysate was added to confluent monolayers at a multiplicity of infection of 1 to 2 and the mixture was incubated for 30 min at 37°C with 5% CO_2. Virus was removed, monolayers were washed with 2 ml of medium, and 4 ml of fresh medium was added. The plates were placed at 37°C with 5% CO_2 for 1 h, after which the medium was changed. Samples (200 μl) from the culture medium were taken at 0, 24, and 48 h postinoculation and assayed for infectious virus by plaque assay on HeLa cell monolayers.

RESULTS

Immunoaffinity protein isolation and amino acid sequencing. Human peripheral mononuclear cells were used as the source for initial immunoaffinity isolation because this preparation contained no connective tissue or other cell types. Whole cells were solubilized with 0.5% DOC, and supernatants derived from 100,000 × *g* centrifugation were passed over an AF3 immunoaffinity column. Silver stain analysis of whole lysate and the immunoaffinity-purified material demonstrated the absence of contaminating protein in the vicinity of the 100-kDa band (Fig. 1A).

For internal amino acid sequencing, the protein was immunoaffinity purified from human spleen tissue. Silver stain analysis of the purified protein revealed the 100-kDa band with several minor contaminants. Western immunoblot analysis of whole spleen and purified protein demonstrated that the lower-molecular-mass bands were not recognized by antibody AF3 (40). Protein sequencing of 20 to 30 pmol of the 100-kDa

Position	1	2	3	4	5	6	7	8	9	10
A. 100kDa/CNBr treated	?	Q	L	V	N	*I*	L	*F*		
		I	?	*L*	F	Q	*G*	S	T	
B. 1st 65kDa/CNBr fragment	?	?	?	*L*	?	*I*	*G*	*F*	?	
C. Final/65kDa CNBr fragment	E	K	A	*L*	S	*I*	*G*	*F*	E	
	(Met) Glu	Lys	Ala	*Leu*	Ser	*Ile*	*Gly*	*Phe*	Glu ...	

FIG 2. Protein sequence comparison. (A) Sequence of 20 to 30 pmol of N-terminal-blocked 100-kDa protein treated, in situ on PVDF, with cyanogen bromide (CNBr), resulting in the appearance of two equimolar sequences. (B) Sequence from 15 to 20 pmol of the isolated 65-kDa fragment that resulted from CNBr treatment of preparatively purified 100-kDa protein. (C) Seventy pmol of ultrapure 65-kDa fragment provided a 28-amino-acid sequence of virtual certainty over 23 positions. The mismatches at other positions of the first two sequences shown are probably due to low levels of signal and to minor impurities in the previous samples.

band, after further purification by preparative SDS-PAGE minigel and electroblotting onto a PVDF membrane, revealed a blocked N terminus. When the 100-kDa protein was treated in situ with cyanogen bromide (CNBr), two equimolar sequences were obtained (Fig. 2A). Treatment of 30 pmol of preparatively purified 100-kDa protein with CNBr resulted in several large fragments (Fig. 1B). Protein sequencing of 15 to 20 pmol of the 65-kDa fragment revealed a match at four positions with the mixed sequence generated by in situ CNBr cleavage of the SDS-PAGE-purified 100-kDa protein (Fig. 2B). Finally, a 70-pmol sample of purified 65-kDa fragment provided a 28-amino-acid sequence of virtual certainty over 23 positions (Fig. 3). This sequence matched both of the previously derived sequences, demonstrating that it was from the 100-kDa protein.

Molecular cloning. Homology search of the SwissProt data base, using the fasta algorithm, demonstrated that the internal amino acid sequence obtained is identical to that of human CDw44a (CD44H) and CD44E and to that of baboon CD44, with a high level of homology to the rat, mouse, and hamster homologs of CD44. In addition, the second partial internal amino acid sequence identified in Fig. 2A consists of four in-phase amino acid matches within the 72-amino-acid cytoplasmic tail, which is found in the major isoforms of human CD44. The internal amino acid sequence shown in Fig. 3 is also common among the isoforms of CD44. However, only cDNA encoding CD44H could be detected by PCR using primers flanking the coding region and lymphocyte cDNA as template (40). Northern (RNA) analysis of lymphocyte RNA previously demonstrated high levels of CD44H but little or no CD44E transcripts (44). In addition, the protein identified by AF3 in Western blots of human lymphocytes, brain, and HeLa cells is identical in size (39, 41). Since all other isoforms of CD44 consistent with amino acid sequence data are 115 to 200 kDa, the 100-kDa protein can only be CD44H. CD44H cDNAs were generated by PCR with lymphocyte cDNA as template and oligonucleotide primers which flank the coding region. Nucleotide sequence analysis of the cloned PCR products confirmed that the inserts encode CD44H.

TABLE 1. Antibody immunoreactivity of transformant cell lines

Cell line	Reactivity[a] with the following antibody			
	AF3	P3H9[b]	A3D8[b]	711C[c]
CD44H-pcDNA1/NEO.33.2[d]	+	+	+	−
CD44H-pcDNA1/NEO.13.1[d]	+	+	+	−
PVR-pcDNA1/NEO.1	−	−	−	+
PVR-pSVL.20B	−	−	−	+
HeLa	+	+	+	+
L	−	−	−	−

[a] Determined by indirect immunofluorescence.
[b] Anti-CD44 monoclonal antibody.
[c] Anti-PVR monoclonal antibody.
[d] Independently derived L-cell transformant.

CD44H cDNA encodes the AF3 epitope. CD44H cDNA was inserted into the mammalian expression vector pcDNA1/NEO and used to derive stable L-cell transformants. Transformants expressing the AF3 epitope were identified by immunofluorescence and subcloned by horseradish peroxidase-enzyme linked immunosorbent assay, with AF3 as probe. Antibody AF3 reacted with mouse L cells stably transformed with CD44H cDNA but not with those transformed with PVR cDNA (Table 1). Anti-CD44 antibodies P3H9 and A3D8 reacted only with CD44H transformants, whereas the anti-PVR antibody 711C reacted only with stable L-cell transformants expressing the PVR. All the antibodies reacted with HeLa cells, and none reacted with L cells.

CD44H cannot act as a PVR. Infectivity and virus binding assays were performed to determine whether CD44H can function as a PVR. L-cell transformants stably expressing CD44H were inoculated with poliovirus, and the yield of infectious virus in cell supernatants was determined at different times after inoculation. Neither the CD44H-L-cell transformants nor normal L cells produced infectious virus by 2 days postinfection (Table 2). Only L-cell transformants expressing the PVR (PVR-pcDNA1/NEO.1 and PVR-pSVL.20B) produced infectious virus in postinoculum supernatants or exhibited cytopathic effect at 4 days postinoculation. Binding of ^{35}S-PV2w2 further demonstrated that neither the CD44H-transformant L cells nor normal L cells bound virus, whereas the PVR-transformant L cells did bind ^{35}S-PV2w2 (40). Therefore, CD44H cannot act as a PVR.

Anti-CD44 antibodies compete with ^{125}I-AF3 for binding to HeLa cells. Previously described anti-CD44 monoclonal antibodies A3D8 and IM7 were tested for the ability to compete for binding with ^{125}I-AF3. Saturating amounts of antibodies were preincubated with the HeLa cell monolayers for 40 min at 37°C, and then ^{125}I-AF3 was added to the monolayers in the presence of the unlabeled antibodies and incubated for an additional 60 min. Saturating levels of AF3, A3D8, and IM7 inhibited ^{125}I-AF3 binding to HeLa cells, while an irrelevant antibody, 5E2B4, had no effect (Fig. 4). Antibody A3D8 inhibits ^{125}I-AF3 binding as well as antibody AF3 does. A3D8

Pos#	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
aa	(Met)	Glu	Lys	Ala	Leu	Ser	Ile	Gly	Phe	Glu	Thr	---	Arg	Tyr	Gly	Phe	Ile	Glu	Gly	His	Val	Val	Ile	Pro

FIG. 3. Partial internal amino acid sequence of a 65-kDa fragment generated by cyanogen bromide treatment of 100-kDa protein preparatively purified by SDS-PAGE. This sequence was generated from a 70-pmol sample, and each position was identified from a 35-pmol signal with no detectable noise. Position 11 corresponds to a cysteine residue in CD44.

TABLE 2. Susceptibility of CD44H transfotmants to poliovirus infection

Cell line	Amt of virus produced[a] at indicated day postinoculation			CPE at day 4[b]
	0	1	2	
CD44H-pcDNA1/NEO.33.2	<1	<1	<1	–
PVR-pcDNA1/NEO.1	<1	4×10^4	5×10^5	+
PVR-pSVL.20B	<1	4×10^4	5.5×10^6	+
L	<1	<1	<1	–

[a] PFU per milliliter on HeLa cells.
[b] Cytopathic effect (CPE) determined by MTT/INT assay (39).

and IM7 were previously generated by using CD44 protein from two different species, human and mouse, respectively, yet the antibodies were subsequently found to bind overlapping epitopes on human CD44 (34). These findings, together with these binding data, suggest that there is a conserved cluster of epitopes that are either highly immunogenic or are involved in specific functions of the CD44 molecule.

Anti-CD44 monoclonal antibodies inhibit binding of radiolabeled poliovirus to HeLa cells. Anti-CD44 monoclonal antibodies A3D8 and IM7 were tested for the ability to inhibit binding of PV2w2 to HeLa cells. HeLa cell monolayers were preincubated with antibodies, and then ^{35}S-PV2w2 was added and the amount of bound radioactivity was determined. The anti-PVR antibody 711C, which blocks poliovirus binding by direct competition for the binding site on the PVR (36), inhibited ^{35}S-PV2w2 binding by 99% (Fig. 5). Antibodies

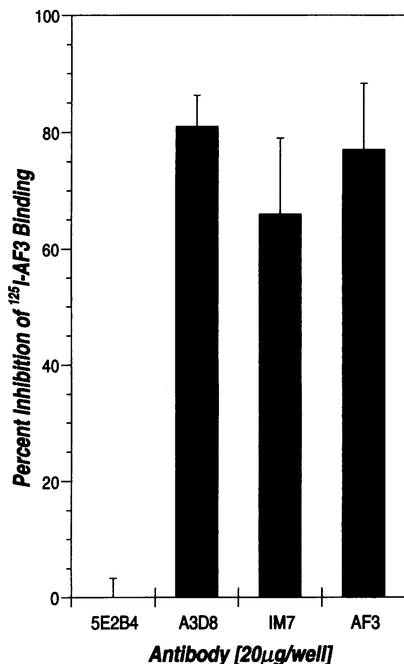

FIG. 4. Competition between ^{125}I-AF3 and anti-CD44 monoclonal antibodies for binding to HeLa cells. All antibodies were preincubated with HeLa cell monolayers at a 400-fold-higher concentration than ^{125}I-AF3. Antibodies A3D8 and IM7 are anti-CD44 monoclonal antibodies. 5E2B4 is an irrelevant monoclonal antibody. ^{125}I-AF3 was added at 10^5 cpm (ca. 50 ng of immunoglobulin) per well. One standard deviation is indicated.

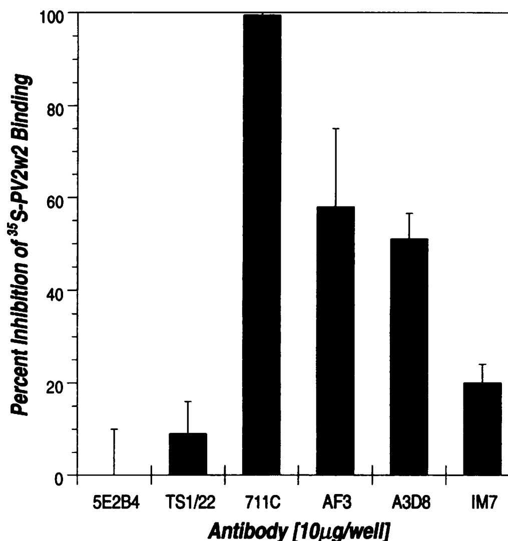

FIG. 5. Inhibition of radiolabeled PV2w2 binding to HeLa cells by monoclonal antibodies. The ability of antibody AF3 to inhibit virus binding to HeLa cell monolayers was compared with that of the anti-CD44 antibodies A3D8 and IM7 and the anti-PVR antibody 711C. 5E2B4 and TS1/22 are irrelevant monoclonal antibodies. All antibodies were preincubated with monolayers, after which 1.5×10^4 cpm of ^{35}S-PV2w2 was added in the presence of antibody. One standard deviation is shown.

A3D8 and AF3 inhibited poliovirus to comparable levels (51 to 58%). Antibody IM7, which was generated against murine Pgp-1, inhibited poliovirus binding to a lesser degree. Antibodies AF3, A3D8, and IM7, therefore, react within a cluster of functionally overlapping or related epitopes on CD44H and alter the ability of the PVR to bind poliovirus.

DISCUSSION

Most strains of poliovirus infect only primates and replicate primarily in the intestines, tonsils, deep cerebellar nuclei, brain stem, motor cortex, and anterior spinal gray matter (3). Results of studies utilizing organ minces and homogenates suggested that the tissue tropism and species specificity of poliovirus are determined primarily by the presence or absence of unique virus binding activity (14). However, examination of the expression pattern of the PVR indicates that a more complex relationship exists between receptor expression and tropism, because PVR mRNA and protein are expressed in a range of tissues, including those that poliovirus does not normally infect (8, 29). Inoculation of transgenic mice expressing the PVR with poliovirus results in replication mainly in neurons in the brain and spinal cord and in skeletal muscle cells, despite expression of PVR mRNA in other sites, including developing T lymphocytes of the thymus, Bowman's capsule, and tubules in the kidney, alveolar cells of the lung, and endocrine cells in the adrenal cortex, intestines and spleen (37). The tissue tropism of poliovirus therefore appears to be controlled by cellular factors other than the PVR, either at the cell surface or in the cytoplasm, that determine the outcome of infection. One possibility is that a second protein may be required, with the PVR, for the entry of poliovirus into susceptible tissues. One such candidate is the protein recognized by antibody AF3, since this protein is involved in poliovirus attachment and it is expressed in human tissues in a manner consistent with poliovirus tissue tropism. Human immunodeficiency virus type

1 appears to require a second human cell factor in addition to the receptor, CD4, for entry of virus into cells. Although expression of human CD4 in a variety of human cell types results in susceptibility to human immunodeficiency virus type 1 infection, murine cells expressing human CD4 cannot be infected, apparently due to a block subsequent to binding (26).

The results reported here demonstrate that antibody AF3, which protects cells from poliovirus infection, recognizes CD44H. Antibody AF3 and the previously described anti-CD44 antibodies P3H9 and A3D8 react with mouse L cells transformed with CD44H cDNA but do not react with L cells transformed with PVR cDNA. The anti-PVR monoclonal antibody 711C does not react with L cells transformed with CD44H cDNA. Binding of ^{125}I-AF3 to HeLa cells, which express CD44, was inhibited by antibodies AF3, A3D8, and IM7. Antibody A3D8 inhibited radiolabeled poliovirus binding as effectively as did antibody AF3, IM7 inhibited virus to a lesser extent, whereas the anti-PVR antibody 711C inhibited poliovirus binding maximally, and two irrelevant antibodies had no effect.

Antibody A3D8 inhibits poliovirus binding as well as does antibody AF3, yet A3D8 binds a wider molecular weight range of CD44 than AF3. Consequently, the same magnitude of inhibition of poliovirus binding is achieved whether a small, specific population of CD44 (that which reacts with AF3) or a much larger population of CD44 (that which reacts with A3D8) is induced to inhibit poliovirus binding. This observation suggests that the small population of specifically glycosylated CD44H species, the 100-kDa species named $_{AF3}$CD44H, is primarily involved in the CD44H-PVR interaction. This hypothesis is particularly interesting given the speculation by some investigators that differential glycosylation may alter the function of subpopulations of CD44 (11). Immunohistochemical data also support the hypothesis that $_{AF3}$CD44H is a functionally distinct species. The anti-CD44 antibody F-10-44-2, which binds the same protein as antibody A3D8 (47), reacts with the white matter of the human brain (28). The Hermes series of antibodies, which also bind to the same CD44 protein (34), react with white matter, the periaqueductal gray matter of the brain stem, the anterior horn gray matter of the spinal cord, and much less so with posterior horn gray matter (48). In contrast, antibody AF3 does not react with the white matter of the human brain stem, but it does react with the periaqueductal motor nuclei of the brain stem as well as with other brain stem nuclei which poliovirus infects (39, 41). Thus, $_{AF3}$CD44H appears to be a functionally distinct species which, in this case, was identified by antibody AF3 because of the ability of $_{AF3}$CD44H to specifically interact with the PVR.

It is interesting that antibodies A3D8 and IM7 inhibit ^{125}I-AF3 binding so well, since the antibodies most likely recognize different epitopes. IM7 and A3D8 bind a wide molecular weight range of CD44 molecules, while antibody AF3 reacts only with a 100-kDa species (39, 41). This observation strongly suggests that the AF3 epitope is present in only a subset of uniquely glycosylated CD44H molecules, whereas IM7 and A3D8 epitopes are present in many of the diversely glycosylated species of CD44H. In further support of this hypothesis, it has been demonstrated that ^{125}I-AF3 binds with high affinity to less than 5% of all CD44H molecules, which amounts to 4,100 AF3 sites per HeLa cell (41). ^{125}I-AF3 binding assays on the CD44H-L-cell transformants reported here demonstrate that AF3 binds to the same small fraction of CD44H molecules, with the same affinity, as was previously determined for HeLa cells (40). If AF3 binds a different epitope than does either antibody A3D8 or IM7, how do A3D8 and IM7 inhibit ^{125}I-AF3 binding? One possibility is that binding of A3D8 and IM7 might result in conformational changes in a spatially distinct AF3 epitope. Alternatively, the AF3 epitope and the A3D8 and IM7 epitopes may overlap. The AF3 epitope might only be present in the 100-kDa species, but the A3D8 and IM7 epitopes might be present in many species of CD44H. When all three epitopes are present simultaneously, as would be the case in the 100-kDa species, antibodies A3D8 and IM7 would be able to inhibit AF3 binding. This hypothesis is particularly interesting since the AF3 epitope would be situated within a cluster of overlapping epitopes identified by antibodies A3D8 and IM7 and by the Hermes 2 antibodies (34) that can induce CD44-mediated modulation of cellular events.

How CD44H interacts with the PVR remains unknown, but there are several possibilities which are consistent with the data. First, CD44H and the PVR may be noncovalently associated. In this case, antibody AF3 or other CD44 antibodies would inhibit poliovirus binding by steric hindrance. It has been suggested that CD44 directly associates with LFA-3 in a noncovalent manner, providing LFA-3, which is anchored to the membrane by a phosphoinositol linkage, indirect anchorage to the cytoskeleton (42). It is not likely that the activity of AF3 results from nonspecific steric hindrance that would occur by binding of an antibody to an abundant cell surface protein, because there are only ca. 4,100 $_{AF3}$CD44H molecules per HeLa cell compared with ca. 100,000 PVR molecules (40). Second, CD44 may be associated with the PVR only after it interacts with poliovirus. Antibodies to CD44 would block subsequent processes leading to viral entry. The PVR protein is sufficient to confer poliovirus binding and susceptibility to mouse cells (29). However, $_{AF3}$CD44H may be required for events subsequent to virus binding, such as penetration or uncoating. If this hypothesis is correct, then a murine homolog of $_{AF3}$CD44H may also provide a similar function. Third, CD44 may interact through other cellular elements without direct association with the PVR. For example, an anti-CD44 antibody has been described that triggers CD44 on T lymphocytes to promote T-cell adhesion through the LFA-1 pathway (25). Although lymphocyte adhesion is mediated through LFA-1, the anti-CD44 induction of adhesion is dependent on protein kinase C and an intact cytoskeleton.

To determine the role of $_{AF3}$CD44H in poliovirus attachment to the PVR, a large number of stable L-cell transformants which express both CD44H and PVR were isolated. Unfortunately, these cell lines could not be used to study the $_{AF3}$CD44H-PVR interaction, because their growth rate was greatly reduced in proportion to the level of expression of both proteins. High-level dual-expressing cells did not survive subcloning. Intermediate expressers could be cloned but quickly lost most surface expression as their growth rate increased. The use of inducible promoter systems did not overcome the growth inhibition, because low levels of promoter activity occurred in the absence of inducer. Other systems are being sought which may be used to determine the nature of the $_{AF3}$CD44H-PVR interaction.

The finding that $_{AF3}$CD44H is involved in poliovirus attachment provides insight into the interaction of the PVR with other cellular components and may facilitate an understanding of the cellular function of the PVR. These results may also lead to an elucidation of the structure-function relationships among the diversely glycosylated group of CD44 molecules and may provide a new opportunity to study the processes underlying CD44-mediated cellular events. The strong correlation of the expression of $_{AF3}$CD44H with poliovirus tropism suggests that the lymphocyte homing receptor is involved in the pathogenesis of poliomyelitis. Finally, these results suggest that

$_{AF3}$CD44H plays a role in the function of the cellular receptor site for poliovirus.

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