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Establishment of an immunoscreening system using recombinant VP1 protein for the isolation of a monoclonal antibody that blocks JC virus infection

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Abstract

Polyomavirus JC (JCV) infection causes the fatal human demyelinating disease, progressive multifocal leukoencephalopathy. Although the initial interaction of JCV with host cells occurs through direct binding of the major viral capsid protein (VP1) with cell-surface molecules possessing sialic acid, these molecules have not yet been identified. In order to isolate monoclonal antibodies which inhibit attachment of JCV, we established an immunoscreening system using virus-like particles consisting of the VP1. Using this system, among monoclonal antibodies against the cell membrane fraction from JCV-permissive human neuroblastoma IMR-32 cells, we isolated a monoclonal antibody designated as 24D2 that specifically inhibited attachment and infection of JCV to IMR-32 cells. The antibody 24D2 recognized a single molecule of around 60 kDa in molecular weight in the IMR-32 membrane fraction. Immunohistochemical staining with 24D2 demonstrated immunoreactivity in the cell membrane of JCV-permissive cell lines and glial cells of the human brain. These results suggested that the molecule recognized by 24D2 plays a role in JCV infection, and that it might participate as a receptor or a co-receptor in JCV attachment and entry into the cells.

Keywords: JC virus; VP1; VLP; Monoclonal antibody; PML

JC virus (JCV), which belongs to the polyomavirus family of non-enveloped DNA viruses, is known to be a causative agent of the progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system (CNS). PML affects mainly immunosuppressed patients, such as those with acquired immunodeficiency syndrome (AIDS) or following organ transplantation, and has increased in prevalence with the spread of transplantations and AIDS. However, there is still no effective treatment for PML. The major target cells of JCV infection in the CNS are the glial cells, such as oligodendrocytes and astrocytes [1]. The first step in the establishment of JCV infection is attachment of the viral capsid to receptors on host cells.

The capsid of JCV is composed of three capsid proteins, VP1, VP2, and VP3. VP1, encoded by the late region of JCV, is the major capsid protein that forms the outer surface of the virion. It has been reported that recombinant VP1 derived from *Escherichia coli* or insect cells can form virus-like particles (VLPs), and exhibit cellular attachment and intracytoplasmic trafficking similar to those of JCV virions [2–5]. In addition, VP1 has been reported to play a major function in the attachment of JCV to cells as the anti-VP1 antibody suppresses viral entry into cells and subsequent infection

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[5]. The cellular receptor molecule for JCV is unknown, but it has been reported to be an N-linked glycoprotein containing α2–6-linked sialic acids, based on the observation that α2–6-specific sialidase inhibits infection of glial cells by JCV. In addition, treatment of permissive cells with tunicamycin, which removes N-linked oligosaccharides, was shown to inhibit JCV infection in vitro [6]. These findings indicate that the interaction between the major structural protein VP1 and sialic acids is critical for JCV infectivity. However, non-enveloped viruses are unable to fuse with the cell lipid membrane bilayer, and their internalization is thought to require a specific entry receptor. Furthermore, it has been reported that infectious entry of JCV requires a ligand-inducible signal that is inhibited by genistein, a protein–tyrosine kinase inhibitor [7]. The mechanisms by which JCV binds and enters host cells and the molecules involved are not yet completely understood.

The mouse polyomavirus (PyV) is also known to bind to the sialic acid residues of a not yet identified receptor. Recently, antibodies to α4β1 integrin have been shown to partially block PyV infection. Therefore, the integrin has been suggested to act as an entry receptor for the early stages of PyV infection in fibroblasts [8].

Antibodies that react with cell-surface molecules have important applications as therapeutic agents and in the identification of viral receptors. Therefore, we attempted to isolate monoclonal antibodies to molecules on JCV-permissive cells. To isolate the monoclonal antibodies against the cell-surface molecules related to JCV infection on permissive cells, we developed an immunoscreening method and attempted to isolate blocking antibodies against JCV infection.

Using this immunoscreening system, we isolated a monoclonal antibody that recognizes the cellular-surface molecules related to JCV infection. The antibody also significantly blocked the attachment and subsequent infection of JCV. The molecule recognized by this antibody was considered to participate in virus infection, suggesting that this molecule acts as a virus receptor and/or a co-receptor. Here, we report the successful isolation and characterization of a monoclonal antibody with blocking activities toward JCV infection.

Materials and methods

Cells and antibodies

The human neuroblastoma cell line, IMR-32 (JCRB 9050), human embryonic kidney cell line, HEK293 (JCRB 9068), and human cervical carcinoma cell line, HeLa (JCRB 9004), were provided by the Health Science Research Resources Bank (Osaka, Japan). The African green monkey kidney cell line, COS-7 (#CRL 1651), was obtained from the American Type Culture Collection (Rockville, MD), and the human glial cell line, SVG-A, was kindly provided by Dr. Atwood [9]. All cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin, Sigma, St. Louis, MO) in 5% CO_2 at 37 °C. The anti-VP1 polyclonal antibody and anti-agnoprotein antibodies were prepared as described previously [5,10]. The anti-SV40T-antigen, which is known to be cross-reactive with anti-JCV T-antigen (Ab-2) monoclonal antibody, was purchased from Calbiochem (San Diego, CA). Anti-actin monoclonal antibody was purchased from Chemicon (Temecula, CA). Anti-GFAP polyclonal antibody (Nichirei, Tokyo, Japan), anti-synaptophysin monoclonal antibody (Dako, Japan), and anti-CA-2 polyclonal antibody (The Binding Site, Birmingham, UK) were prepared commercially. A mouse IgM-isotype control antibody was obtained from Sigma (St. Louis, MO).

JC virus and recombinant virus-like particles

Preparation of native JCV was performed as described previously [5]. Briefly, cell lysates from the JCV-producing cell line, JCI cells, were harvested, subjected to repeated freeze–thaw cycles, and then treated with 0.05 mg/ml neuraminidase type I (Sigma) at 37 °C for 16 h. After treatment with neuraminidase, samples were incubated at 56 °C for 30 min and centrifuged at 1000*g* for 10 min. The viral titer of virus-containing supernatant was quantified by hemagglutination (HA) assay and stored at −80 °C until use.

The preparation of virus-like particles (VLPs) was performed as described previously [5]. Briefly, the VP1 gene of JCV from pBR-Mad1 [11] was subcloned into the prokaryotic expression vector pET15-b (Novagen, Madison, WI). The plasmid was transformed into *E. coli*, BL21 (DE3)/pLys (Stratagene, La Jolla, CA). The expression of VP1 was induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30 °C, and the mixture was centrifuged at 4000*g* for 10 min. The cell pellet was resuspended in reassociation buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1.0 mM $CaCl_2$) containing 1 mg/ml lysozyme, kept on ice for 30 min, and then sodium deoxycholate was added to a final concentration of 2 mg/ml. After 10 min on ice, the sample was lysed by five cycles of sonication in 15-s bursts. The lysate was treated with DNase I (100 U/ml) for 30 min at 30 °C and centrifuged at 10,000*g* for 10 min.

The supernatant containing the VP1 protein was subjected to 20% sucrose cushion centrifugation at 100,000*g* for 3 h at 4 °C. The pellet was resuspended with reassociation buffer and purified by CsCl gradient centrifugation. The peak fraction of VP1 (density 1.29 g/ml) was then collected and dialyzed against the reassociation buffer. The morphology of VLP, which has a diameter of 45 nm, was confirmed by electron microscopy. VLPs were quantified by HA assay, as described previously [12].

Preparation of cell membrane fraction

IMR-32 cells were harvested, washed with PBS, and centrifuged for 3 min at 1000*g*. The cells were suspended in 5 volumes of a solution containing 0.25 M sucrose and 20 mM Hepes (pH 7.5), and disrupted with a glass Dounce homogenizer. The nuclei and unbroken cells were removed by centrifugation for 7 min at 1000*g*. The supernatant was transferred to a new centrifugation tube and pelleted at 105,000*g* for 1 h in a Hitachi P70AT rotor at 4 °C. The pellet was resuspended with 3 ml of a buffer containing 2.1 M sucrose and 20 mM Hepes (pH 7.5), and purified by sucrose gradient centrifugation. The peak fraction of the membrane fraction was collected and centrifuged for 1 h at 105,000*g*. The pellet was resuspended with 1 ml of a buffer containing 20 mM Tris–HCl (pH 7.5), 400 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 1 mM PMSF in the presence of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM DTT) and incubated at 4 °C for 30 min. The protein concentration was quantified by Bio-Rad (Hercules, CA) assay using bovine serum albumin as a standard.

Production of monoclonal antibodies against IMR-32 cell-membrane protein

BALB/c mice were immunized five times with 100 μg IMR-32 cell membrane fraction, emulsified in a Freund's complete adjuvant (Sigma) for primary immunization and in a Freund's incomplete adjuvant (Sigma) for four further immunizations at 7-day intervals. Dot-blot analysis and enzyme-linked immunosorbent assay (ELISA) were performed to confirm the immunoreactivity of sera against the IMR-32 cell membrane fraction. The mice that showed recognition of IMR-32 cell membrane fraction were boosted with a final immunization, and 4 days later the spleen cells from immunized animals were fused to P3U1 myeloma cells to produce approximately 600 hybridoma-containing wells.

Development of the immunoscreening system

We developed an immunoscreening system to isolate a monoclonal antibody that recognizes the JCV receptor from antibodies raised against IMR-32 cell membranes (Fig. 1A). As it is difficult to obtain sufficient amount of native JCV, we used VLPs instead of the native virion for immunoscreening.

A 96-well microtiter plate (Nalgen Nunc International, Rochester, NY) was coated with purified IMR-32 membrane fraction (1 μg/well) in 100 mM sodium bicarbonate buffer (pH 9.5). As a negative control, the wells were coated with BSA or left uncoated. Non-specific binding was blocked with PBS (pH 7.4) containing 25% Block Ace and 0.05% NaN_3. Aliquots of 200 μl of supernatant from each hybridoma were loaded onto each membrane-coated well, and the microtiter plate was incubated overnight at 4 °C. After washing three times with PBS, a solution of purified VLPs (5000 HA) was added to each well, followed by incubation at 37 °C for 1 h. The unbound VLPs were washed out with PBS. The plates were then incubated with a 1:1000 dilution of anti-VP1 antibody in EC buffer (20 mM phosphate buffer, pH 7.0, containing 400 mM NaCl, 2 mM EDTA, 10% Block Ace, 0.2% BSA, 0.05% NaN_3, and 0.075% Chaps) at room temperature for 2 h. After washing with PBS, a 1:5000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio Source International, Camarillo, CA) in C buffer (20 mM phosphate buffer, pH 7.0, containing 400 mM NaCl, 2 mM EDTA, 10% Block Ace, 0.2% BSA, and 0.1% thimerosal) was added, and the plates were incubated for 3 h at room temperature. After washing three times, 100 μl of TMB reagent (Pierce Chemical, Rockford, IL) was added and developed for 15 min before quenching with an equal volume of 1 M phosphate buffer. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad, Laboratories, Hercules, CA). In this system, antibodies that recognize VLP binding protein will inhibit binding of VLP to the membrane-coated well, while antibodies that do not recognize VLP binding protein will permit binding of VLP to the well.

To confirm the specificity of VLP binding to the IMR-32-coated wells, we examined the binding kinetics. Initially, we determined how much VLP was appropriate for detection of bound VLPs to the membrane-coated wells. The amounts of VLPs bound to the wells increased in a dose-dependent manner from 500 to 1×10^4 HAU, and reached saturation at a concentration of 1×10^5 HAU in both 0.5 and

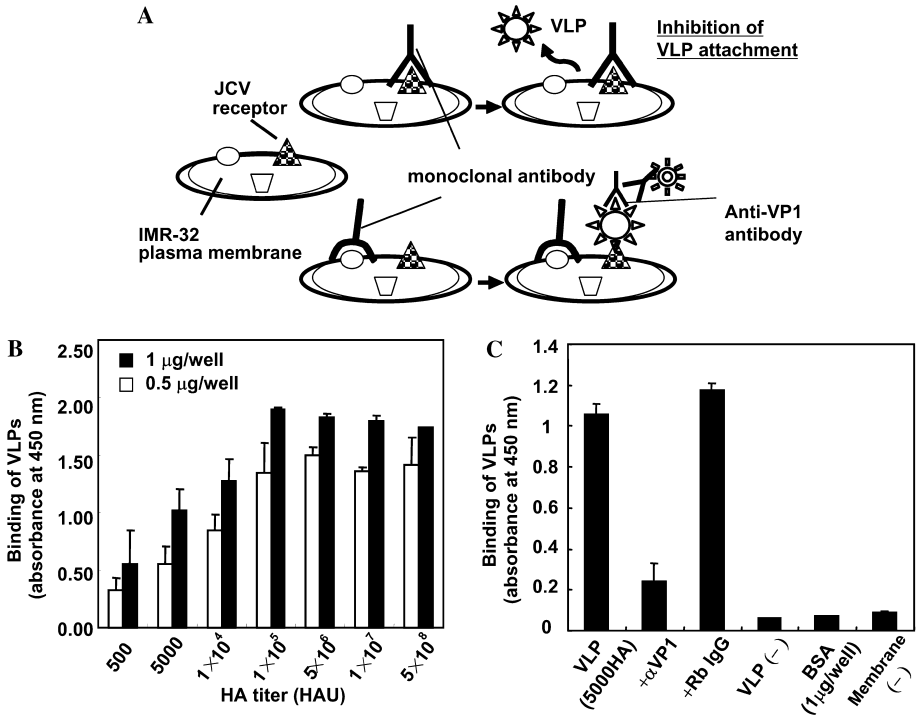

Fig. 1. Development of the immunoscreening system. (A) Strategy of the immunoscreening system using a blocking antibody against JCV infection. BALB/c mice are immunized with the membrane fraction from the human neuroblastoma cell line, IMR-32 cells. The wells of a 96-well plate are coated with the membrane fraction of IMR-32 cells, and then incubated with monoclonal antibodies derived from immunized mice before addition of VLPs. The amount of bound VLPs to the IMR-32 membrane fraction coated onto the wells is quantified with anti-VP1 antibody, followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins and TMB reagent. Absorbance was measured at 450 nm with a microplate reader. (B) The kinetics of binding of VLPs to IMR-32 membrane fractions. The amount of bound VLPs to the membrane fraction increased in a dose-dependent manner and peaked in the presence of 1×10^5 HAU VLPs. (C) Specificity of binding of VLPs to membrane-coated wells. The binding of VLPs was significantly inhibited in the presence of anti-VP1 antibody that recognizes JCV viral capsid protein, VP1, but was not affected in the presence of the isotype control rabbit normal IgG (Rb IgG). Negative controls, including VLP (−), BSA, and membrane (−), did not bind to the wells. The bars represent the standard deviation of the mean of at least three independent experiments performed in duplicate.

1 μg IMR-32 membrane protein-coated wells (Fig. 1B). In addition, a relatively low level of non-specific binding was recognized as compared with the negative control, and VLP binding was inhibited significantly in the presence of anti-VP1 antibody with application of 5000 HAU VLPs (Fig. 1C). Thus, it was demonstrated that VLPs bound to IMR-32 membranes in a specific manner in this immunoscreening system.

After immunoscreening of more than 600 clones, one clone, designated as "24D2," was identified as one of the antibodies showing the greatest inhibitory effect. The subtype of 24D2 was shown to be IgM using a monoclonal antibody isotype detection kit (Amersham–Pharmacia Biotech, Piscataway, NJ). The hybridoma cells were recloned to confirm the production of a monospecific antibody and cultured in a CELLine culture system (BD Biosciences, San Jose, CA) to obtain a large amount of the antibody. The concentration of the resulting supernatant was measured by semi-quantitative immunoblotting assay using mouse immunoglobulin as a standard. The antibody was separated by SDS–PAGE on a 10% gel. After blocking with 5% skim milk/PBST for 30 min, the anti-mouse immunoglobulins were diluted 1:5000 in PBST. Immunoreactive bands were detected by ECL (Amersham–Pharmacia Biotech) and analyzed with LAS-1000 Plus image analyzer (Fuji Film, Tokyo, Japan). The concentration of 24D2 was determined as the intensity relative to that of the control antibody.

Inhibition of VLP binding to IMR-32 cells by 24D2

For conjugation of purified VLPs with fluorescein isothiocyanate (FITC), aliquots of 2 mg of purified VLPs were dissolved in 0.1 M carbonate–bicarbonate buffer (pH 9.0), mixed with 156 μg FITC (Sigma), and incubated at room temperature for 2 h. To eliminate unlabeled FITC, the samples were centrifuged at 100,000*g* for 1 h at 4 °C. The pellet was dissolved in PBS, and centrifuged at 10,000*g* overnight, and the pellet was resuspended in PBS.

Before the experiment, IMR-32 cells (2×10^4) were plated onto 8-microwell tissue culture chambers (Nalgen Nunc International) and cultured in DMEM containing 10% FBS. The cells were pre-incubated in the absence or presence of either 24D2 (50 μg/ml) or isotype control monoclonal antibody for 1 h at 37 °C. Subsequently, FITC-VLPs (1×10^5 HAU) diluted 1:10 with DMEM were added to each chamber for 1 h at 4 °C. Unbound FITC-VLPs were removed by washing with PBS, and incubated for 15 min at 37 °C, following observation with a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Inhibition of JCV infection by 24D2

IMR-32 cells (5×10^4) were preincubated in the absence or presence of either 24D2 (300 μg/ml) or the isotype control monoclonal antibody for 1 h at 37 °C. The cells were then incubated with 512 HAU JCV in fresh medium with 2% FBS for 1 h at 37 °C. After washing twice with fresh medium, the cells were incubated at 37 °C for 4 days with DMEM supplemented with 2% FBS. The monolayer culture in each well was washed with PBS and lysed in 1% Triton X-100/TBS containing 2 μg/ml aprotinin. The cell lysates were centrifuged at 4 °C for 10 min at 15,000 rpm, and the supernatants were separated by SDS–PAGE on 12% gels. After transferring the separated proteins onto a PVDF membrane, the membrane was immersed in 5% skim milk/TBS-T (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 30 min, and subsequently incubated with either anti-agnoprotein antibody or anti-T-antigen antibody diluted 1:1000 with TBS-T for 1 h. After washing twice with TBS-T, the membrane was incubated with HRP-conjugated $F(ab')_2$ goat anti-rabbit or HRP-conjugated $F(ab')_2$ goat anti-mouse immunoglobulins diluted 1:5000 in TBS-T for 1 h. Immunoreactive bands were detected using the ECL reagent (Amersham–Pharmacia Biotech) and analyzed using an LAS-1000 plus image analyzer. Expression of viral proteins in the cells is represented as intensity relative to that of the control sample without treatment with 24D2 or isotype control antibody. The data are presented as mean values ± SD of three independent experiments.

Analysis of the molecule recognized by 24D2

Immunoblotting. Immunoblotting was performed as described above with the following modifications. The purified IMR-32 membrane fractions were separated by SDS–PAGE on 10% gels, transferred onto PVDF membranes, and immunoblotted with the 24D2 antibody. Immunoreactive bands were analyzed as described above.

Immunocytochemistry. IMR-32 cells grown on 8-microwell chambers were fixed with methanol at −80 °C for 2 min and rehydrated in PBS for 5 min at room temperature. After incubation with blocking solution (PBS containing 3% BSA), cells were incubated with 24D2 antibody diluted 1:60 in blocking solution for 2 h. After washing three times with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgM antibody (Molecular Probes, Eugene, OR) diluted 1:500 for 1 h, and observed using a laser scanning confocal microscope (Olympus).

Immunohistochemistry. Normal human brain tissue obtained at autopsy was sectioned at a thickness of 10 μm with a Cryo 2000 cryostat (Sankyo, Tokyo, Japan) and fixed in acetone at 4 °C for 10 min. The sections were rinsed in PBS, blocked in 0.3% H_2O_2 methanol for 15 min, and incubated with 24D2 at 4 °C overnight. After incubation with biotinylated second antibody, immunoreactive products were visualized with 3,3′-diaminobenzidine. For double immunostaining of human brain tissue, the sections were rinsed twice in PBS, preincubated in PBS containing 3% BSA for 60 min, and incubated with anti-glial fibrillary acidic protein (GFAP, 1:5 dilution), anti-carbonic anhydrase-II (CA2, 1:1,000 dilution), or anti-synaptophysin (1:100 dilution) together with 24D2 (1:5) overnight. The sections were incubated with the appropriate secondary antibodies, including Alexa Fluor 488-conjugated goat anti-rabbit IgG, biotinylated anti-sheep IgG, Alexa Fluor 488-conjugated streptavidin, Alexa Fluor 488-conjugated goat anti-mouse IgG, or Alexa Fluor 594-conjugated goat anti-mouse IgM (Molecular Probes). After rinsing with PBS for 30 min, the sections were observed by laser scanning confocal microscopy.

Results

Isolation of monoclonal antibody (24D2) which inhibits attachment of JCV

Among more than 600 clones of monoclonal antibodies against the cell membrane fraction of JCV-permissive human neuroblastoma cell line IMR-32 cells, one hybridoma clone was isolated using the immunoscreening system and was designated as "24D2" (Fig. 1A). 24D2 significantly inhibited the binding of VLPs (Fig. 2A) as well as JCV (Fig. 2B) to cell membrane fractions on the immunoplates, whereas an isotype control antibody (mouse IgM) showed no inhibitory effect (Figs. 2A and B).

The 24D2 inhibits attachment and entry of FITC-labeled VLPs into IMR-32 cells

To evaluate the inhibitory effects on attachment and entry of VLPs, which have functions in cellular attachment and entry, similar to native JCV infection

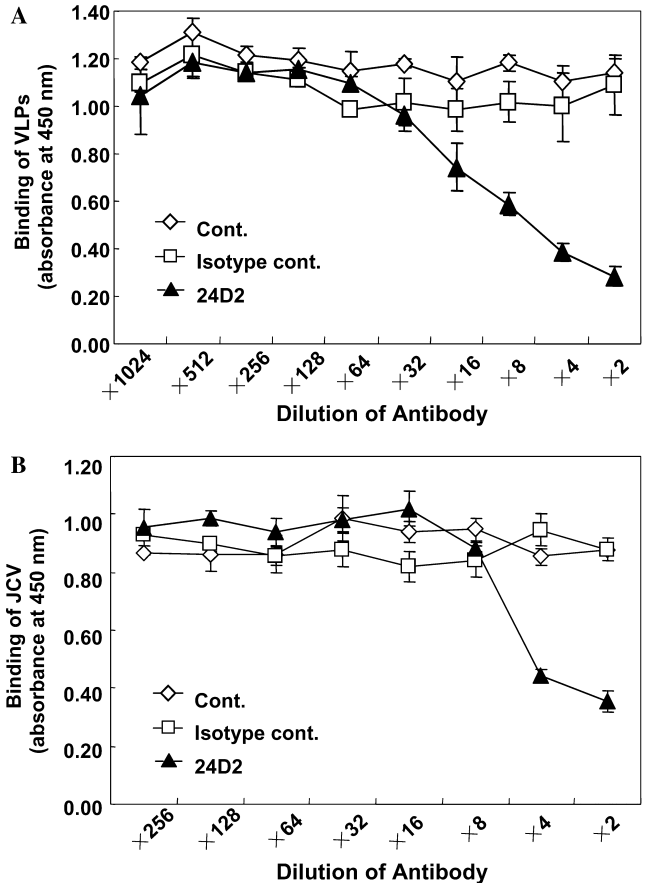

Fig. 2. Inhibition of the binding of both VLPs and JCV to IMR-32 membrane fractions on the immunoplate system by 24D2. (A,B) Inhibition of JCV and VLP binding by 24D2. IMR-32 membrane fractions were coated onto each well of the immunoplates, which were preincubated with serial dilutions of 24D2 (▲), mouse IgM (isotype control antibody, □), or without antibody (◊), and then incubated with 5000 HAU of VLPs (A) or JCV (B). Binding of VLPs or JCV was detected with anti-VP1 antibody and presented as the absorbance value. The bars represent the standard deviation of the mean of at least three independent experiments performed in duplicate.

[2,6] into IMR-32 cells, the cells were pretreated in the presence of either 24D2 or the isotype control monoclonal antibody, and incubated with FITC-labeled VLPs. 24D2 completely inhibited cellular attachment and entry of FITC-VLPs in IMR-32 cells, while the isotype control antibody had no effect on attachment or entry (Fig. 3).

Inhibition of JCV infection to IMR-32 cells by 24D2

To determine the blocking activity of 24D2 against JCV infection, IMR-32 cells were incubated with 24D2 before JCV inoculation. Four days after inoculation, expression of the viral proteins, including early and late viral proteins, T-antigen, and agnoprotein, was examined by immunoblotting. Treatment with 24D2 markedly inhibited expression of both T-antigen and agnoprotein in JCV-infected cells (Figs. 4A and B). The inhibitory effect of 24D2 against JCV infection was dose-dependent (Fig. 4C). Thus, we demonstrated that 24D2 possessed inhibition activity against JCV infection, suggesting that the molecule recognized by 24D2 plays a role as a cellular receptor for JCV.

Characterization of the molecule recognized by 24D2

We further characterized the molecule recognized by 24D2 by immunoblotting. 24D2 clearly recognized a single band with a molecular weight of around 60 kDa in membrane fractions from IMR-32 cells (Fig. 5A). Immunoblotting was also performed using various cell lines, including, HEK-293, HeLa, COS7, and SVG-A. In HEK293 cells, 24D2 recognized a single band with the same molecular weight as that of IMR-32 cells. However, in HeLa, COS7, and SVG-A cells, positive signals were observed around 30 kDa instead of 60 kDa (Fig. 5B).

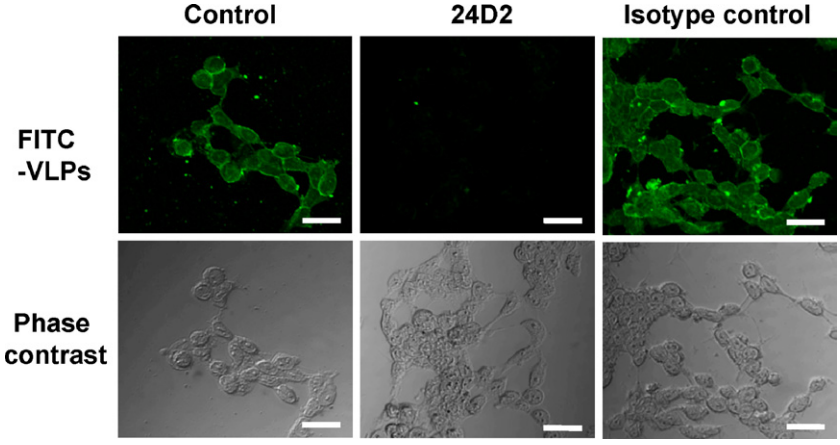

Fig. 3. Inhibitory effect of 24D2 on attachment and entry of FITC-labeled VLPs (FITC-VLPs) into IMR-32cells. After 1-h incubation with either 24D2 or the isotype control antibody, IMR-32 cells were incubated with FITC-VLPs. The immunofluorescence signal of FITC-VLPs was observed by confocal microscopy. No FITC signal was observed in cells treated with 24D2. Bars, 20 μm.

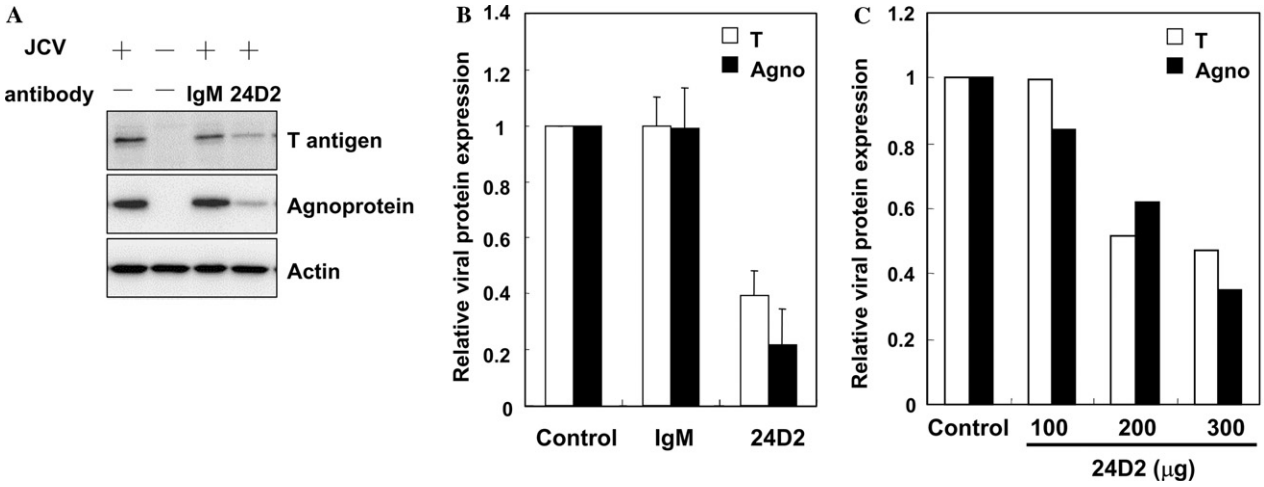

Fig. 4. Inhibition of JCV infection by treatment with 24D2. IMR-32 cells pretreated in the absence or presence of 24D2 or the isotype control antibody (IgM) were inoculated with 512 HAU of JCV for 1 h. Four days after inoculation, the cells were harvested and analyzed with immunoblotting using specific antibodies for JCV proteins (large T-antigen and agnoprotein). (A) Representative immunoblotting data with antibodies for large T-antigen and agnoprotein. Anti-actin antibody was used as a loading control. (B) Expression levels of viral proteins in IMR-32 cells treated with 24D2 or the isotype control antibody (IgM) relative to non-treated IMR-32 cells (control) at 4 days after inoculation of JCV. The bars represent mean values ± SD of at least three independent experiments. (C) The dose-dependent inhibition of JCV infection by 24D2. IMR-32 cells were incubated with different concentrations of 24D2 and incubated with 512 HAU JCV. JCV T-antigen and agnoprotein expression in IMR-32 cells was analyzed by immunoblotting 4 days after inoculation. The data are presented as amounts of viral protein expression relative to that of control infection. The assays were performed at least twice independently.

Next, we investigated the subcellular localization of positive signals with 24D2 in IMR-32 and HEK-293 cells by immunocytochemistry. The immunopositive signals for 24D2 were detected mainly on the cell surface and in the intracytoplasmic compartment (Fig. 5C). We performed the immunocytochemical examination using HeLa, COS, and SVG cells. In these cells, the immunopositive signals against 24D2 were also recognized in the cell surface and in the cytoplasm (data not shown).

The molecule recognized by 24D2 is localized mainly in glial cells in the human brain

Recently, it has been reported that JCV receptor-type α2–6-linked sialic acid was localized in oligodendrocytes and astrocytes but not in cortical neurons [13]. Although the viral genome was detected in various organs, such as the kidney, lymphoid tissue, lung, liver, and intestinal tract, the major target cells of JCV infection in the CNS are glial cells, such as oligodendrocytes and astrocytes [14,15]. Therefore, we investigated the subcellular localization of the molecule recognized by 24D2 in the human brain. Positive signals for 24D2 were distributed in the glial cells of the cerebrum and cerebellum (Fig. 6A). To confirm the cellular distribution of the immunopositive signals, we performed double immunofluorescence staining using 24D2 and cell-specific markers, such as CA2, as a marker of oligodendrocytes [16–19], GFAP, a marker of astrocytes, and synaptophysin, a neuronal marker (g–i) (Fig. 6B). The immunopositive signals of 24D2 were colocalized with GFAP and CA2 (Figs. 6Ba–f). However, 24D2 failed to react with synaptophysin-positive neurons (Fig. 6Bg–i). These results indicated that the molecule recognized by 24D2 is distributed mainly in glial cells in the human brain.

Discussion

Virus attachment to cell-surface molecules is an initial event in the process of virus infection. Many viruses utilize various receptors for attachment and entry into cells. The polyomavirus, simian vacuolating virus 40 (SV 40), binds to the MHC class I molecule at the cell surface [20], but the MHC molecule is not internalized into the cells together with SV 40 [21]. The ganglioside GM1 also binds to SV 40, and is mediated to transport the virus from the plasma membrane to the endoplasmic reticulum (ER), and a role of GM1 as a functional receptor has been suggested [22]. The mouse polyomavirus (PyV) is also known to bind to the sialic acid of an as yet unidentified receptor [23]. Efforts to identify the receptor by screening for monoclonal antibodies that can protect cells from infection have been unsuccessful [24]. It has been reported that an antibody to α4β1 integrin partially inhibits PyV infection [8]. Therefore, the integrin has been suggested to act as an entry receptor in the early stages of PyV infection in fibroblasts, and PyV utilizes gangliosides as carriers from the plasma membrane to the ER [22]. Interestingly, SV40 and PyV have been shown to use clathrin-independent and caveolin-

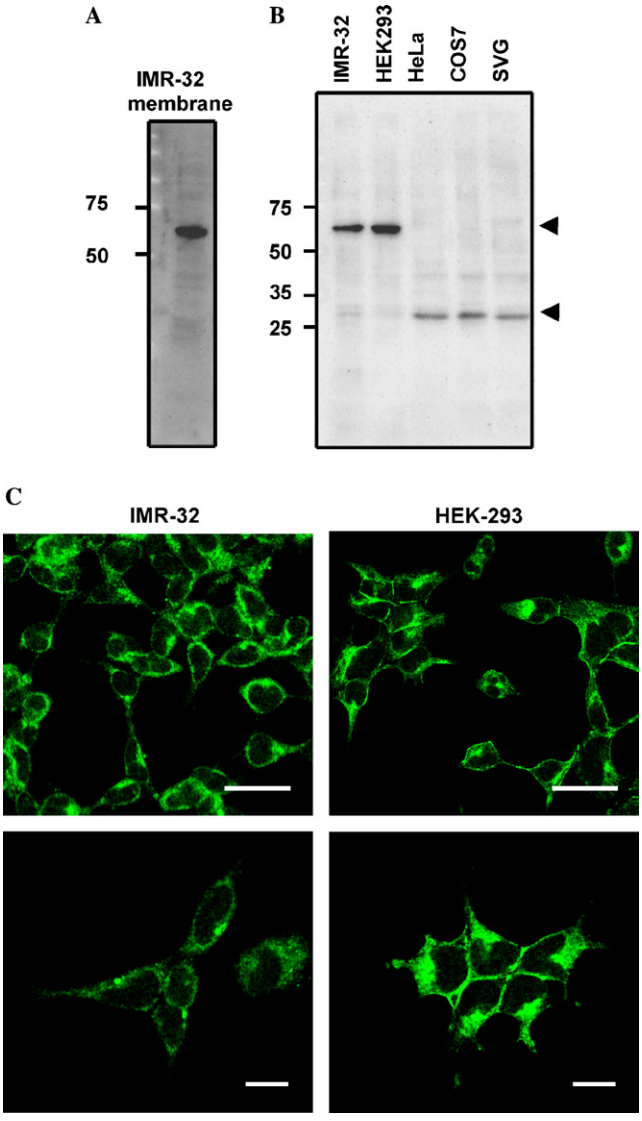

Fig. 5. Characterization of the molecule recognized by 24D2. (A) Immunoblotting analysis with membrane extracts derived from IMR-32 cells. Aliquots of 20 μg of the detergent-solubilized IMR-32 membrane fraction were loaded and immunoblotted with 24D2 at a dilution of 1:60. (B) Immunoblotting analysis with various cell lysates. The membrane fractions were prepared from IMR-32, HEK293, COS7, HeLa, and SVG-A cells. Molecular size markers are indicated on the left of the column. (C) Subcellular localization of the molecule recognized by 24D2 in IMR-32 and HEK-293 cells. Methanol-fixed cells were incubated with 24D2 at a dilution of 1:60, following Alexa Fluor 488-conjugated goat anti-mouse IgM. The immunofluorescence signal is represented as a green color. The lower panels show higher magnifications of the upper panels. Bars, 20 μm (upper panels) and 10 μm (lower panels).

dependent mechanisms to infect cells, respectively [25–27]. In contrast, JCV enters host cells by receptor-mediated clathrin-dependent endocytosis [28]. Furthermore, it has been reported that JCV does not share receptor specificity with SV 40 on human glial cells, because anti-class I antibodies failed to inhibit JCV infection [6].

It has been reported that a sialic acid-containing glycoprotein is one of the receptors of the human polyomavirus JCV in human glial cells, as neuraminidase treatment suppressed JCV attachment to the cells and subsequent infection [6].

In the present study, we have shown that the monoclonal antibody 24D2 inhibits JCV infection in IMR-32 cells. Although the major targets of JCV infection in the central nervous system are the glial cells, the viral genome was detected in various organs, such as the kidney, lymphoid tissue, lung, liver, and gastrointestinal tract [29–31], and JCV can enter a wide variety of cell types [5]. The 60-kDa molecule recognized by 24D2 was detected not only in IMR-32 cells, but also in other cell lines, including the human kidney cell line, HEK293. In addition, 24D2 showed inhibition of cellular attachment and entry of FITC-VLP to HEK293 cells (data not shown). These results suggest that the molecule recognized by 24D2 also acts as a receptor in IMR-32 and HEK293 cells. As 24D2 failed to completely inhibit infection, additional molecules might participate in JCV attachment and infection.

It is interesting to note that JCV has been suggested to persistently infect the kidney, because JCV genome was detected in the urine and the renal tissue of healthy individuals [32,33]. 24D2 also recognized a molecule of 30 kDa in the membrane fractions of HeLa, COS7, and SVG-A cells. This smaller molecule might be an isotype or a proteolytic product of the larger molecule. It has been reported that a monoclonal antibody against the hyaluronan receptor recognized two distinct receptors in the cell membranes of rat liver sinusoidal endothelial cells, which were identified previously as hyaluronan-binding proteins of 175 and 300 kDa [34,35]. After reduction of disulfide bonds, the 175-kDa hyaluronan receptor was shown to be a single protein, whereas the 300-kDa molecule consisted of three subunits, i.e., α (269 kDa), β (230 kDa), and γ subunits (97 kDa) [35], and it has been reported that the 175-kDa receptor was derived from the 300-kDa receptor by proteolytic processing [36]. Similar to the hyaluronan receptors, the molecules of 30 and 60 kDa recognized by 24D2 might be isoreceptors for the same ligand or products derived from a larger receptor complex. It has been reported that the receptor with 30-kDa molecular weight for human coronavirus OC43 was isolated from the newborn mouse brain membrane fractions [37]. A 30-kDa molecule detected in HeLa, COS7, and SVG cells might be similar to a coronavirus OC43 receptor.

We also examined the localization of the putative receptor molecule in the human brain, because JCV infects mainly glial cells in the central nervous system. Positive immunoreactivity for 24D2 was colocalized with CA2-positive oligodendrocytes and GFAP-positive astrocytes, but failed to colocalize with synaptophysin-positive neurons. These findings indicated that the putative receptor molecule for JCV was localized mainly in the glial cells of the human brain. In the present study,

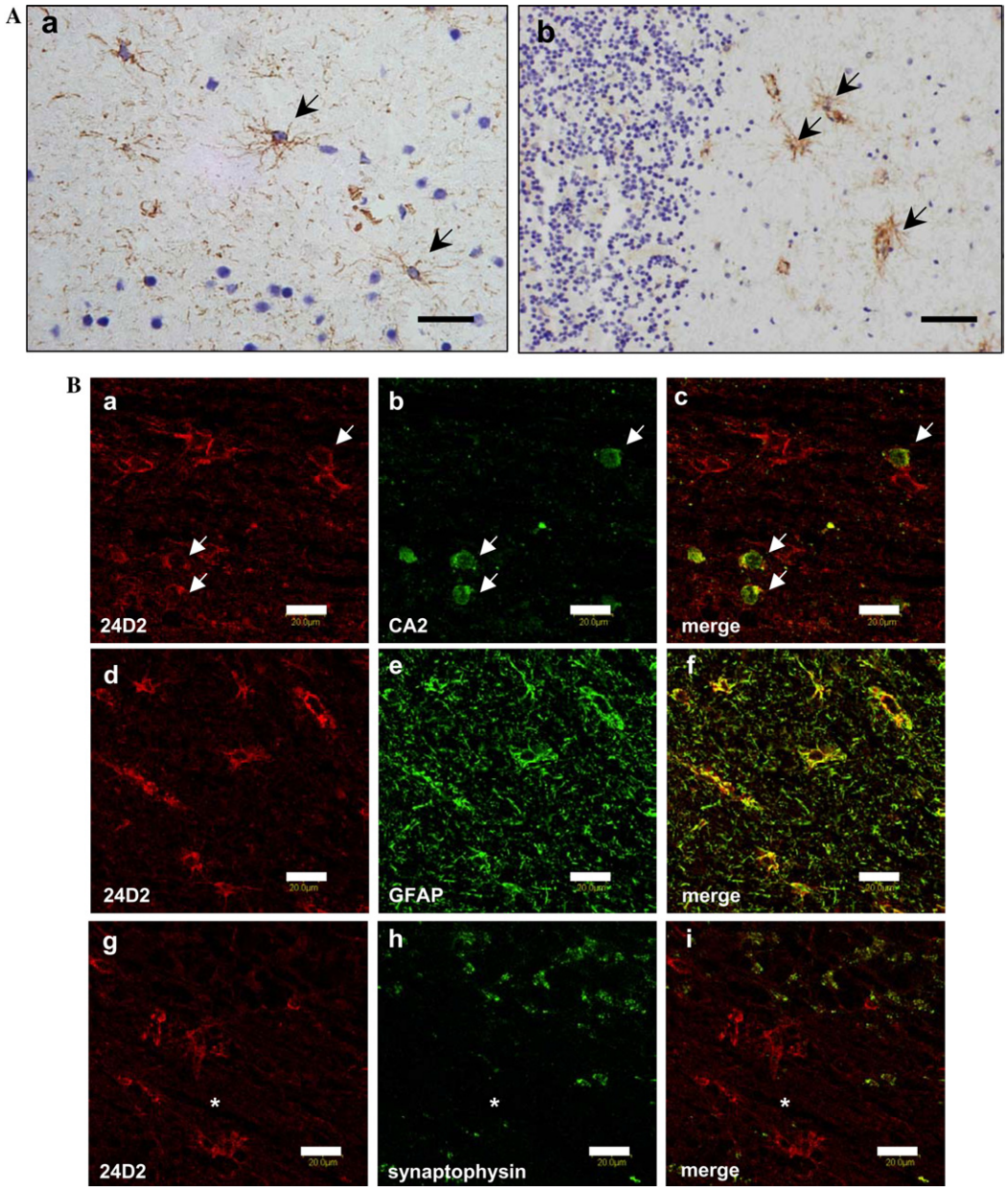

Fig. 6. Subcellular localization of the molecule recognized by 24D2 in the human brain. (A) Immunoreactivity with 24D2 in the cerebrum (a) and the cerebellum (b). Arrows indicate the immunopositive cells (a,b) in the white matter. Scale bars, 50 μm. (B) Double immunostaining using 24D2 and anti-CA2 antibody (a–c), anti-GFAP antibody (d–f), or anti-synaptophysin antibody (g–i) in the cerebrum. The merged images show that the molecule recognized by 24D2 (red color) was colocalized with CA2 and GFAP (green color). However, colocalization was not detected between 24D2 and anti-synaptophysin signals. Arrows indicate the 24D2- and CA2-double positive cells (a–c). Asterisks indicate white matter (g–i). Scale bars, 20 μm.

the neuroblastoma cell line, IMR-32, was used as the immunogen for the monoclonal antibody because these cells are highly susceptible to JCV infection [38]. Furthermore, immunization of IMR-32 cell has been shown to produce an antibody that specifically recognizes a molecule in oligodendrocytes [39]. Thus, it was suggested that IMR-32 cells possess molecules in common with brain glial cells. In Fig. 6Aa and b, the immunopositive signals against 24D2 were less than those of Fig. 6Ba, d, and g. We suppose that the difference of oligodendrocyte 24D2 immunostaining patterns among Fig. 6A and B might be due to the applied immunostaining systems. In Fig. 6A, we applied the biotinylated antibody as a second antibody, and the sections were observed with light microscopy. Meanwhile, in Fig. 6B, we observed the sections labeled with Alexa Fluor 488-conjugated second antibodies with fluorescent microscopy.

In summary, we have isolated a monoclonal antibody against the receptor molecule for JCV. By a newly established immunoscreening system, we isolated a monoclonal antibody (24D2) that inhibits cellular attachment

and infection of JCV. We also showed that the antibody specifically recognizes a membrane molecule with a molecular weight of 60 kDa, and positive signals were observed in the glial cells of the human brain. These results suggest that the molecule recognized by 24D2 acts as a JCV receptor in JCV infection. Further studies are required to characterize and determine the functional role of the molecule. A greater understanding of the molecular characteristics of the receptor molecule is necessary for the design of novel anti-JCV agents. Experiments to identify the molecules detected by 24D2 using a λ phage library are currently in progress in our laboratory.

Note added in proof

Recently, it has been reported that the antagonists and antibodies against the 5HT2 serotonergic receptors blocked JCV infection in SVG-A cells [40], suggesting that serotonin receptors act as one of the JCV receptors.

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