The Neural Cell Adhesion Molecule Is a Receptor for Rabies Virus

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Previous reports strongly suggest that, in addition to the nicotinic acetylcholine receptor, rabies virus can use other, as-yet-identified receptors. We found that laboratory cell lines susceptible to rabies virus infection express the neural cell adhesion molecule (NCAM) (CD56) on their surface, whereas resistant cells do not, supporting the idea that NCAM could be a rabies virus receptor. We observed that (i) incubation with rabies virus decreases the surface expression of NCAM; (ii) treatment of susceptible cells with heparan sulfate, a ligand for NCAM, or with NCAM antibodies significantly reduces the rabies virus infection; and (iii) preincubation of rabies virus inoculum with soluble NCAM protein as a receptor decoy drastically neutralizes the capacity of rabies virus to infect susceptible cells. Moreover, we demonstrated that transfection of resistant L fibroblasts with the NCAM-encoding gene induces rabies virus susceptibility whereas absence of NCAM in the primary cortical cell cultures prepared from NCAM-deficient mice reduces the rabies virus infection and virus production. This provides evidence that NCAM is an in vitro receptor for the rabies virus. Moreover, the in vivo relevance for the use of NCAM as a receptor was demonstrated by the infection of NCAM-deficient mice, in which rabies morality was delayed and brain invasion by rabies virus was drastically restricted. Our results showed that NCAM, which is expressed mainly in the adult nervous system, plays an important role in rabies infection. However, it cannot be excluded that receptors other than NCAM are utilized. Thus, the description of NCAM as a new rabies virus receptor would be another example of the use by viruses of more than one receptor to gain entry into the host.

The rabies virus (RV) is an enveloped bullet-shaped virus of the Rhabdoviridae family, genus Lyssavirus. The viral particle is constituted of a membrane made of host lipids and two proteins, G and M, surrounding a helical nucleocapsid (NC). The trimeric G protein is the only protein exposed on the surface of the virion. It is responsible for the attachment to the target cell by interaction with several cell membrane components. Following the attachment of G to the cell membrane, the RV enters the cell by endocytosis. Fusion of the viral membrane with endosomal membranes liberates the NC into the cytosol, where transcription and replication occur. Although RV can infect various types of cells, especially in laboratory culture conditions, its main target is the neuron. The RV binds to carbohydrates, phospholipids, and gangliosides and to protein receptors including the nicotinic acetylcholine receptor (AchR) (4). Lentz et al. observed that RV accumulates at neuromuscular junctions and colocalizes with α-bungarotoxin binding (21). They observed that preincubation of fixed chick myoblasts with α-bungarotoxin or d-tubocurarine, both of which specifically compete with acetylcholine for the AchR α subunit, and with a monoclonal antibody (MAB) against the α subunit of AchR reduced the number of myotubes infected by RV. The same group recently used a virus overlay binding assay to confirm that RV binds to the α subunit of AchR (12). These findings strongly suggest that AchR may serve as an RV receptor. However, the capacity of the RV to infect stretch proprioceptors, sensory endings, and several cell lines that do not express AchR implies that RV can use other, as-yet-unidentified receptors (39). The identification of any such receptors would be a significant contribution to our understanding of how the RV infects cells.

The recent finding that lymphocytes support RV infection suggests that these cells also express an RV receptor (37). There is evidence that neurons and lymphocytes have common surface molecules, particularly cytokine and chemokine receptors and adhesion molecules, which have been reported to be used by viruses and parasites to infect the cells (2, 17, 28). Assuming that the RV receptor is a member of this category of molecules, we extensively surveyed the surface molecules on RV-susceptible and nonsusceptible cell lines. All RV-susceptible cell lines have the neural cell adhesion molecule (NCAM) on their surface, whereas it is not found on the surface of resistant cell lines. NCAM, also called CD56, D2CAM, Leu19, or NKH-1, is a cell adhesion glycoprotein of the immunoglobulin (Ig) superfamily which has been described for neurons, astrocytes, myoblasts, myotubes, activated T cells, and NK cells (13, 20). It is encoded by a single gene which is very well conserved among vertebrate species. Three major isoforms of NCAM, with molecular masses of 120, 140, and 180 kDa, are generated by alternative mRNA splicing (35). They all contain an almost identical ectodomain composed of five Ig-like and two type III fibronectin-like domains. The major differences among the three isoforms are in their transmembrane and cytoplasmic domains: only NCAM-120 is linked to the membrane via a phosphatidylinositol-glycan transmembrane anchor, and NCAM-140 and NCAM-180 have cytoplasmic tails of different lengths. Additional diversity among these subtypes is generated by alternative splicing of transcripts encoded by several small exons, resulting in differences in ectodomain size and glycosylation. All three forms can be posttranslationally modified by addition of polysialic acid, a carbohydrate moiety.
The goal of the work was to determine the role of NCAM in RV infection both in vitro and in vivo. We assayed in vitro the effect of NCAM blockade by ligands and the neutralization of virus by soluble NCAM. We also analyzed RV infection of cells transfected with genetic constructs encoding various NCAM isoforms (-140 or -180) (15). The role of NCAM in vivo was investigated by using knockout mice lacking all three isoforms of NCAM (9). We demonstrate that NCAM is a receptor for the RV. Thus, the RV can utilize at least two different receptors, AChR and NCAM, to invade the host.

MATERIALS AND METHODS

Cells and viruses. Mouse NCAM-negative L cellblasts and stable L-NCAM-negative cells expressing the full-length mouse NCAM isofrom, D9 and A14 cells (15), and laboratory cell lines were grown as described in the literature. RV laboratory strain CVS (challenge virus standard) was obtained from the American Type Culture Collection, Manassas, Va. (no. v959). Vaccinia virus was a gift from Robert Dr理想信念. RV and vaccinia virus were propagated on BSR cells, and cell culture supernatants were used as inocula. RV was also purified from supernatants of infected cells on a sucrose gradient and concentrated as previously described (37). For 35S labeling of the RV, cell culture medium was replaced 2 days after infection by methionine-free minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Transfected L cells were incubated on a sucrose gradient, and concentrated. Cells and viruses were mycoplasma-free as determined by the plaque assay technique.

Antibodies (MAbs and reagents). Texas red-conjugated streptavidin, MEM, RPMI, Ham’s F-12 medium, and methionine-free MEM were obtained from Gibco BRL (Cergy-Pontoise, France). Phycoerythrin-conjugated streptavidin was from Dako. Biotin-conjugated anti-Ig mouse goat Ab and the ECL kit were purchased from Amersham (Little Chalfont, Buckinghamshire, United Kingdom). Mouse MAb directed against mouse NCAM (CD56), rat MAb directed against the s6 chain of integrin (CD49) or CD9 molecules, and a mouse polyclonal Ab directed against human CD56 were obtained from TransGenic (Marnes la Coquette, France). Purified recombinant soluble NCAM protein, containing all 5 Ig domains and the two type III fibronectin-like molecules, and a mouse polyclonal Ab directed against human CD56 were obtained from PharMingen (San Diego, Calif.). The rat H28 purified MAb (IgG2a), directed against mouse brain tissue-extracted NCAM, was a gift from Christo Goridis (16). The rat IgG2a MAB (anti-V6 T-cell receptor) used as the isotype matching control was from the laboratory. Fluorescein isothiocyanate (FITC)-conjugated NC-specific rabbit Ab was purchased from Serotec (Dartmouth, United Kingdom). As a control, a rabbit Ab directed against NCAM (IgG3) or with a rat MAb directed against NCAM (H28) or against an irrelevant protein (anti-V6 T-cell receptor) in 50 µl of MEM-2.5% FCS for 30 min at 4°C. Abs were washed before incubation with FITC conjugated anti-NCAM IgM and Texas red- or phycoerythrin-conjugated streptavidin with the ECL kit.

Virus neutralization. To analyze the effect of soluble NCAM protein on RV neutralization, BSR cells were infected with viral inoculum preincubated with soluble NCAM or with control proteins (Ig anti-human CD3 and laminin). Inocula containing 13 µg of concentrated RV or vaccinia virus (as a control) were preincubated with 0.7 to 1.5 µg of purified NCAM for 1 h at 4°C, washed to remove the viral inoculum and incubated at 37°C under 5% CO2. Neutralization of RV infection was quantified by the plaque assay technique (33% plaque-reduction or 100% plaque-reduction). To analyze the effect of NCAM on RV infectivity, the preincubation was extended for 1 h at 37°C (50 µl of concentrated RV or vaccinia virus). Neutralization of RV infection was also determined by the plaque-reduction or 100% plaque-reduction. To analyze the effect of NCAM on RV infectivity, the preincubation was extended for 1 h at 37°C (50 µl of concentrated RV or vaccinia virus). Neutralization of RV infection was also determined by the plaque-reduction or 100% plaque-reduction. To analyze the effect of NCAM on RV infectivity, the preincubation was extended for 1 h at 37°C (50 µl of concentrated RV or vaccinia virus). Neutralization of RV infection was also determined by the plaque-reduction or 100% plaque-reduction.
TABLE 1. Infection of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>RV susceptibility</th>
<th>Presence of NCAM on surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR-32</td>
<td>Human neuroblastoma</td>
<td>100</td>
<td>+ + +</td>
</tr>
<tr>
<td>N2a C1300</td>
<td>Mouse neuroblastoma</td>
<td>100</td>
<td>+ + +</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney cells</td>
<td>100</td>
<td>+ + +</td>
</tr>
<tr>
<td>CER</td>
<td>Chicken embryoid related (or hamster)</td>
<td>80–100</td>
<td>+</td>
</tr>
<tr>
<td>BSR and BHK-21</td>
<td>Hamster kidney cells</td>
<td>80–100</td>
<td>+</td>
</tr>
<tr>
<td>NIH 3T</td>
<td>Embryonic mouse fibroblast</td>
<td>80–100</td>
<td>+</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Hamster ovary</td>
<td>80–100</td>
<td>+ + +</td>
</tr>
<tr>
<td>WEHI 7.1</td>
<td>Mouse lymphoma</td>
<td>100</td>
<td>+ + +</td>
</tr>
<tr>
<td>L-929 and L cells</td>
<td>Mouse fibroblast</td>
<td>1–5</td>
<td>–</td>
</tr>
<tr>
<td>YAC-1</td>
<td>Mouse lymphoma</td>
<td>1–5</td>
<td>–</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human epithelioid carcinoma, cervix</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>HeEp-2</td>
<td>Human epidermoid carcinoma, larynx</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>MRC5</td>
<td>Human lung</td>
<td>30</td>
<td>± (faint fluorescence in only a few cells)</td>
</tr>
<tr>
<td>PC12</td>
<td>Rat adrenal pheochromocytoma</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>NFG-treated PC12</td>
<td>Rat adrenal pheochromocytoma</td>
<td>28</td>
<td>+'</td>
</tr>
</tbody>
</table>

* Measured as the percentage of cells infected 24 h after initial contact with virus. Permeabilized cells were stained with anti-rabies NC rabbit Ab conjugated with FITC.

* Surface NCAM was revealed with either anti-human CD56 Ab or an anti-mouse CD56 MAb depending on the origin of the cells. Anti-mouse and anti-human CD56 Abs cross-react with hamster and rat and simian NCAM, respectively.

* Forty-three percent.

Statistical analysis. Differences between NCAM-deficient and wild-type mouse groups were analyzed by appropriate frequency analysis including Fisher’s exact test and Student’s t test.

RESULTS

RV infects NCAM-positive cells. Cell lines of human-simian, chicken, or rodent (mouse, rat, and hamster) origin were tested for their susceptibility to the rabies CVS strain at an MOI of 1. Percentages of cells infected 24 h after initial contact were recorded (Table 1). The cell lines could be classified into two groups. The first group consisted of susceptible cell lines in which infection spread rapidly (80 to 100% of the cell population infected within 24 h). A second group was composed of RV-resistant cell lines: less than 10% of the total cells infected after 24 h. The human MRC5 cell line was intermediate, with 30% of cells infected. We tested whether different susceptibilities corresponded to differences in the viral binding. Aliquots (5 × 10⁵ N2a and L cells) were incubated for 1 h at 4°C with 15,000 cpm of [³⁵S]metabolically labeled RV, and 2,968 ± 269 cpm bound to N2a whereas only 1,536 ± 241 cpm bound to L cells (significant at *P = 0.003*) (data not shown).

The cell lines were tested for surface expression of NCAM by an anti-NCAM MAb staining technique. NCAM was found on the surface of the susceptible cell lines (IMR-32, N2a, Vero, BSR, CER, CHO-K1, WEHI 7.1, NIH 3T3, and MRC5 cells) but not on that of the resistant cell lines (L cells, L-929, YAC-1, HeLa, HeEp-2, and undifferentiated PC12) (Table 1). Note that only 20% of the MRC5 cells, a line poorly susceptible to infection (30%) were stained, suggesting that rabies susceptibility correlated with the level of NCAM expression in the cell population. To assess this possibility, we used nerve growth factor (NGF) supplementation, a treatment which upregulates NCAM production in PC12 cell cultures (10). PC12 cells were supplemented with NGF (50 ng/ml for 48 h). NCAM was consequently produced in 43% of cells in the culture. This was paralleled by increased susceptibility to RV (28% of NGF-treated PC12 cells were infected compared to 8% of the undifferentiated PC12 cells). Thus, the susceptibility of cultivated cell lines to RV correlates with NCAM expression.

In the experiments described below, we used three cell types as model systems to study the role of NCAM in RV infection: N2a, BSR, and L cells. The RV-susceptible, mouse neuroblastoma N2a line expresses both NCAM and nicotinic AchR, the baby hamster kidney BSR cells express NCAM only, and the non-RV-susceptible, mouse fibroblast L cells do not produce either NCAM or AchR.

RV decreases surface expression of NCAM. We analyzed the effect of an incubation with RV on the expression of NCAM on the cell surface of N2a and BSR cells. Cells were intoxicated at 37°C for 30 min with a series of doses of RV (10 and 30 infectious particles per cell) or PBS (mock treated). NCAM surface expression was compared in the three populations by cytofluorimetry with an anti-NCAM Ab (Fig. 1). Exposure to the virus decreased the intensity of fluorescence, and the effect was dose dependent: 61.4 mean fluorescence in mock-treated BSR cells, 55.5 in BSR cells treated with 10 PFU per cell, and 33.3 in BSR cells treated with 30 PFU per cell. The findings for NCAM fluorescence in N2a cells were similar (Fig. 1C). This decrease in NCAM fluorescence intensity was not observed if the virus and BSR or N2a cells were incubated at 4°C (data not shown), a temperature that does not allow internalization despite virus attachment to the cells. The fact that this phenomenon was observed only at a physiological temperature is consistent with the binding of virus to the cell surface triggering internalization of NCAM surface molecules.

We analyzed the effect of vaccinia virus on the surface NCAM on BSR cells, which are also susceptible to this virus (Fig. 1B). Incubation of BSR cells with vaccinia virus does not affect surface NCAM, indicating that the loss of surface NCAM is RV specific. We then analyzed the effect of RV on the surface behavior of other integral proteins of the N2a cell membrane, the α6 chain of integrin and CD9. The treatment of N2a cells with RV in the conditions that resulted in the loss of NCAM (Fig. 1C) did not modify the amount of α6 chain of integrin molecules on the surface (Fig. 1D). Similarly, RV did not affect the expression of CD9 molecules on the surface of BSR cells (data not shown).

These observations indicate that treatment with RV does not trigger a general perturbation of the membrane structure and suggest that RV specifically modulates the surface expression of NCAM. Since RV enters cells by an adsorptive endocytosis pathway, the partial disappearance of NCAM from the cell surface may well be the consequence of the internalization of NCAM-virus complexes in endocytosis vesicles.
Heparan sulfate and Ab directed against NCAM inhibit RV infection. We analyzed whether blocking NCAM decreased cell susceptibility to RV infection. We first tested the effect of heparan sulfate, a physiologically relevant natural ligand of NCAM (7). NCAM-positive cells (BSR or N2a cells) and negative cells (L cells) were either mock treated (PBS), or treated with heparan sulfate or with chondroitin sulfate A, B, or C. Chondroitin sulfate A, B, and C were used as controls for non-specific inhibition resulting from the charge characteristics of the glycosaminoglycan. At a nontoxic concentration (10 μg/ml), heparan sulfate inhibited RV infection by up to 60% in N2a cells and 55% in BSR cells but only by 13% in L cells (Fig. 2A). Unexpectedly, unlike chondroitin sulfate A and B, chondroitin sulfate C strongly inhibited RV infection in BSR cells (53%) and in NCAM-negative L cells (43%). To our knowledge, chondroitin sulfate C has not been reported to be an NCAM ligand and the mechanism of the antagonist effect has not been described yet.

To confirm the role of NCAM in virus entry, we treated N2a and BSR cells with polyclonal Ab directed against NCAM or with control Ab before infection. NCAM-specific Abs were raised against a native NCAM preparation (1). NCAM Ab pretreatment inhibited infection of N2a cells by 60% (Fig. 2B, left part) as well as that of BSR cells (data not shown), whereas rabbit preimmune Ab or irrelevant Ab had no effect. Similar findings were obtained when BSR cells were treated with a rat MAb directed against NCAM (H28 MAb). As shown in the right part of Fig. 2B, pretreatment of BSR cells with H28 MAb strongly inhibited RV infection (49% inhibition of infection with 10 μg of H28 and up to 76% with 20 μg of H28 MAb).

A pretreatment of N2a cells with an anti-CD49f MAb directed against the α6 chain of integrin, or with an isotype control MAb, did not modify the RV N2a susceptibility. This indicates that the NCAM Ab-mediated inhibition was NCAM specific. These results indicate that occupancy of cell surface NCAM by Ab or heparan sulfate can block RV infection of cells expressing NCAM. This strongly supports the hypothesis that NCAM is a receptor for RV.

Soluble NCAM neutralizes RV infection. Our next approach was to test whether treatment of RV with a soluble NCAM preparation neutralized the virus particles. Viral inocula were incubated with a series of doses of soluble NCAM (0.7 to 1 μg) or with medium alone and assayed for their residual capacity to infect BSR cells (Fig. 3). Pretreatment of RV with concentrations of NCAM dose dependently reduced (up to 100%) the capacity of RV to infect cells (Fig. 3) and reduced virus production by infected cultures by a factor of 1,000-fold (3-log reduction in the virus titers) (data not shown). The NCAM dose-effect curve was not linear (Fig. 3), indicating a two-step reaction, consistent with the fact that G molecules are associated in trimers on the surface of RV. In contrast, NCAM (0.75 or 1 μg) pretreatment of vaccinia virus did not decrease its capacity to infect BSR cells (right part of Fig. 3), indicating that NCAM treatment was not toxic either for the cells or for the viral inoculum. Neither laminin (even at 100 μg) a component of the basement membrane, nor an irrelevant protein had any effect (extreme left of Fig. 3), indicating that the neutralization effect was NCAM specific.

These observations suggest that RV G, the virion surface protein responsible for the attachment of virus particle to cells, has a fixation site for NCAM, nor an irrelevant protein had any effect (extreme left of Fig. 3), indicating that the neutralization effect was NCAM specific.

Transfection of resistant L cells with NCAM cDNA induces RV susceptibility. We tested whether transfection of resistant cells with NCAM cDNA resulted in susceptibility to RV. Four different cell lines were used: L cells transfected with NCAM genes coding for NCAM-180 (D9 cells) or -140 (cell line A14), N2a cells that produce all three isoforms (180, 140, and 120), and negative RV-resistant L cells (see Western blotting anal-
ysis in Fig. 4). Cytofluorimetric analysis of NCAM expression in the four types of cells revealed differences in intensity and distribution of fluorescence. NCAM was detected on the surface of 79% of N2a, 89% of D9, and only 55% of A14 cells (Fig. 4). NCAM surface fluorescence intensity was weaker on NCAM-transfected cells (mean fluorescence of 62.9 and 34.7 for D9 and A14 cells, respectively) than on N2a cells (mean of 142). Thus, unlike N2a cultures in which the majority of cells were brightly NCAM fluorescent (36% of the population had a fluorescence index higher than 141), strongly positive NCAM cells constituted only a small fraction of transfected cell populations (8.2 and 4% of cells in lines D9 and A14, respectively, had a fluorescence index higher than 141).

The susceptibility of the transfected cells to RV infection was compared to those of nontransfected control L and N2a cells by immunofluorescence analysis (microscopy and cyto-

FIG. 3. Soluble recombinant NCAM protein neutralizes RV infection. RV and vaccinia virus inocula were incubated with 0.7 to 1 μg of soluble NCAM or with control proteins (irrelevant Ig domain and laminin) for 40 min at 37°C. The effect of the different treatments was estimated by measuring the residual infectious virus expressed as a percentage of BSR cells infected after 18 h of culture (left part for RV and right part for vaccinia virus). Values are the means of four separate experiments ± standard deviations.

Fluoresent of the viral inoculum

FIG. 4. Characteristics of NCAM-transfected cells. Expression of NCAM was analyzed by Western blotting and cytofluorimetry in N2a, NCAM-negative L cells transfected with either the plasmid (control cells) or NCAM-180 (D9 cells) or NCAM-140 (A14 cells) isoform cDNA, with a MAb directed against an epitope common to the three NCAM isoforms. The solid line indicates the fluorescence profile obtained with NCAM MAb. The left peak represents FITC-conjugated secondary Ab binding. Bars and numbers represent the gated regions and percentages of cells expressing NCAM, respectively.

To elucidate further the role of NCAM expression in susceptibility to RV, experiments involving blocking RV infection with heparan sulfate and anti-NCAM Ab were performed. Heparan sulfate treatment inhibited 37.5 and 33.3% of infection in A14 and D9 cells, respectively (Fig. 5D), confirming that the acquired susceptibility of the transfected cells was a result of the expression of NCAM and not of the transfection manipulation.

Thus, transfection with NCAM cDNA partially restores sus-
FIG. 5. RV susceptibility of transfected NCAM cells. (A and B) Two-day cultures of RV-infected N2a, NCAM-transfected (D9 and A14), and NCAM-negative (control) cells were double stained for NCAM (red) and viral NC (green) and examined by microscopy (A) or analyzed by flow cytometry (B). RV-infected NCAM-positive cells appear as yellow-stained cells in the leftmost A panels and are located in the upper right quadrant in the B panels. (C) RV susceptibility of D9 (dashed curve), A14 (solid black curve), and control (dotted curve) cells was assessed by infection with RV (MOI of 1.5 to 0.25). Results are expressed as the percentages of cells infected 48 h after infection. (D) Inhibition of RV infection of control, D9, A14, and N2a cells by heparan sulfate and chondroitin sulfate A and B (left, middle, and right bars in each set, respectively).
ceptibility to RV, and NCAM-transfected cells appear to become preferential targets once they express a certain amount of NCAM on their surface. These results support the previous observation that RV susceptibility correlates with NCAM expression (Table 1) and strongly suggest that there is a minimum density of NCAM which allows virus infection.

The absence of NCAM reduces the susceptibility of primary cortex cultures to RV. Day 3 primary cortex cultures were prepared from individual early postnatal wild-type and NCAM-deficient littermate mice (newborn to 2 days old). After 3 days of maintenance in vitro, they were assayed for their susceptibility to CVS infection at an MOI of 10. RV infection was evaluated by (i) the percentage of RV NC-positive cells in each culture 4 days after infection and (ii) the virus produced and released into day 7 cortex culture supernatants. The NCAM phenotype of each cortex culture was determined by testing for NCAM expression by allele-specific PCR analysis and by immunocytochemistry with an anti-NCAM MAb. Horizontal bars indicate the mean values for infection and virus production per a definite number of cells in culture ± standard deviations.

FIG. 6. RV infection of primary cortical cultures from NCAM-deficient and wild-type mice. Three-day cortical cell cultures prepared from postnatal wild-type or NCAM-deficient mice were infected with CVS at an MOI of 10 and cultivated for 3 more days. Percentages of infected cells (A) and virus production per cell in culture (B) were determined for 11 NCAM-positive and 7 NCAM-negative cortical cell cultures. NCAM-positive and NCAM-negative cortical cell cultures were tested for NCAM expression by allele-specific PCR analysis and by immunocytochemistry with an anti-NCAM MAb. Horizontal bars indicate the mean values for infection and virus production per a definite number of cells in culture ± standard deviations.

Extensively distributed in the brains of NCAM-deficient mice. In particular, the cortex sections were only faintly and scarcely fluorescent in NCAM-deficient mice whereas they were strongly positive in wild-type mice (Fig. 7A). The amounts of RV NC that accumulated in three different parts of the brain were compared in wild-type and NCAM-deficient mice. Nervous tissue suspensions of three parts of the brain (cerebellum plus brain stem, diencephalon, and cortex) were assayed for N protein antigen by ELISA (Fig. 7B). The suspension of cerebellum plus brain stem from NCAM-deficient mice contained fourfold-less N protein than did the wild-type cerebellum (514 ng/ml versus 2,310 ng of N protein per ml of nervous tissue suspension). Diencephalon and cortex from NCAM-deficient mice contained 16-fold less rabies antigen than those of wild-type mice (14 ng/ml versus 234 ng of N protein/ml of nervous tissue [P < 0.005]). These measures indicate that, after infection into the masseter muscles, the RV invades brains of NCAM-deficient mice much less efficiently than those of wild-type mice and that progression of infection is severely impaired after infection of the cerebellum and brain stem.

RV mortality is delayed in NCAM-deficient mice. We investigated whether the absence of NCAM interfered with RV infection in vivo. Adult wild-type and NCAM-deficient mice were injected with rabies CVS in the muscle of both hind legs, and the kinetics of RV-induced mortality were monitored. Nine days after inoculation, 50% of the wild-type mice were dead, whereas all the NCAM-deficient mice have survived. In the NCAM-deficient group, 50% mortality was reached only at 13 days postinjection. The mean length of survival was 13.6 days for NCAM-deficient mice and 10 days for wild-type mice (Fig. 7C). This 4-day delay in NCAM-deficient mice is consistent with the restricted viral invasion of the brain reported in Fig. 7A and B. This suggests that the absence of NCAM limits the virus progression in vivo and delays, but does not prevent, the rabies death.

DISCUSSION

We report strong evidence that NCAM expression plays an important role in cell susceptibility to RV both in vitro and in vivo. We show that (i) transfection of NCAM-negative cells (L cells) with the NCAM gene significantly enhances susceptibility to RV, (ii) specific blocking (antibody or natural ligand) or removal (primary cortical cell cultures prepared from NCAM-deficient mice) of NCAM drastically reduces cell susceptibility to RV, and (iii) preincubation with soluble NCAM protein as a decoy substantially decreases the capacity of RV to infect cells. We demonstrated that NCAM is a receptor for RV in vitro. The marked differences in the infection pathways and the slower progression of the disease in NCAM-deficient mice were consistent with these in vitro results and indicate that NCAM plays a significant role in vivo.

NCAM appears early in the development of all germ layer tissues. In later histogenesis, this molecule is mainly associated with muscle formation and the development of the nervous system. During the perinatal period, NCAM-positive cells include neurons and glial, skeletal, cardiac, and kidney cells as well as muscle fibers (31). The tissue distribution of NCAM is more limited in the adult: NCAM persists in most neurons and astrocytes in the central nervous system and on neural cell bodies, their nonmyelinated axons, and nonmyelinating Schwann cells in the peripheral nervous system (27). NCAM-140 is also found in adult hematopoietic tissues, where it is known as the human leukocyte differentiation antigen, CD56 (Leu19/NKH-1). CD56 is predominantly expressed on natural killer cells, in peripheral blood T lymphocytes, and in immature bone marrow cells (20). RV susceptibility of the laboratory cell lines
investigated was entirely consistent with the expression of NCAM in these tissues and developmental stages. Embryos of chicken or duck origin and suckling mouse brains have been used for decades as tissues for the production of RV vaccine. Adaptation of wild strains of RV to N2a cells and to suckling mouse brain is also very efficient. Similarly, RV vaccines were or are still prepared with cells of kidney origin such as fetal bovine kidney cells, Vero cells (African green monkey cells), and, for experimental or veterinary uses, baby hamster kidney cells (BHK-21). All these cells, as can be predicted from the organ origin, are indeed highly positive for NCAM. In contrast, most fibroblast cell lines are NCAM negative and resistant to

FIG. 7. RV infection and mortality in NCAM-deficient mice. (A and B) RV infection in the brains of wild-type and NCAM-deficient mice, 6 days after inoculation of the masseter muscle, was assessed by detecting RV NC by immunofluorescence (A) and N protein by immunocapture ELISA (B). (A) RV infection in cortex sagittal slices of NCAM-deficient (top) and wild-type (bottom) mice with FITC-conjugated anti-NC Ab. Bars represent 5 μm. (B) Production of RV N protein in three parts of the brain, cerebellum plus brain stem, diencephalon, and cortex, of wild-type (black bars) and NCAM-deficient (gray bars) mice. Numbers represent the N protein concentrations (picograms per milliliter) of tissue suspension. (C) Day of death according to the phenotype of mice. The mean day of death was 10.2 for the wild-type group and 13.6 for the NCAM-deficient group. Each group included eight animals. The difference was significant (P = 0.002).
cell-cell contact where it may be involved in stabilization of isoforms in the N2a cells. The flexibility of the two isoforms is this may be due to the presence of three different NCAM effects N2a cells more efficiently than NCAM-transfected cells. This may result in a more efficient RV uptake: we showed that RV in- teractions or stabilization of one isoform by the other could reagents for RV infection suggests that the virus binding site is located in the first two Ig-like domains of NCAM. This conclusion need to be confirmed, for example, by mapping experiments to rule out the possibility that heparan sulfate binding has a steric occupancy effect or causes conformational changes in NCAM (8). Possibly, identifi- cation of the RV binding site in the first two Ig-like domains may help to elucidate the mechanisms by which RV induces neuronal dysfunction. NCAM is not only involved in cell rec- ognition but is also capable of transducing recognition events. In particular, it has been shown that the stimulation of Ig-like I and/or II domains induces intracellular changes of inositol phosphate metabolim, Ca\(^{2+}\) flux, and pH (3, 23, 36). The binding of RV to a receptor that is also a signaling molecule may modify the metabolism of the neuronal cell target. This may contribute to neuronal perturbations or the behavioral changes that characterize rabies.

The membranes of hamster kidney cells, BHK-21 cells, contain a protein complex which is a receptor for RV but which has not yet been identified (33). This receptor was described as an RV-specific entity that functions independently of Ca\(^{2+}\) and Mg\(^{2+}\). It consists of a doublet of high-molecular-weight protein and of at least four other proteins migrating between 66 and 200 kDa (4). The high-molecular-weight protein carrying the specific binding activity was described as an integral glyco- sylated membrane component and as being sensitive to pro- nase and subtilisin enzymatic treatment. We assessed whether NCAM does not belong to this complex receptor. We found that BHK-21 cells express NCAM. Moreover, both major iso- forms of NCAM (-140 and -180) are glycosylated integral membrane proteins and are sensitive to pronase and subtilisin. NCAM binding activity does not require Ca\(^{2+}\) or Mg\(^{2+}\). The properties shared by NCAM and the BHK-21 receptor, as well as the evidence that BHK-21 cells and their derived clone, BSR cells, use an NCAM-dependent pathway for RV infection, sug- gest that a glycosylated NCAM isoform is the main or even the only component of the BHK-21 receptor. However, the NCAM pathway is not the only mediator of RV penetration into kidney cells: RV infection can develop even in the o- called resistant cell lines (10% compared to 80 to 100% in NCAM-positive cells). This alternative route of entry was not inhibited by heparan sulfate or by preincubation with an anti- NCAM Ab. The nature of the NCAM-independent pathway used by the RV to infect kidney cells is unknown, but the path- way may involve a nonprotein receptor (4). Our data suggest, however, that this pathway is sensitive to chondroitin sulfate C treatment.

There is strong evidence that nicotinic AchR, especially \(\alpha\)-bungarotoxin-sensitive nicotinic AchR at the muscular junction, can be used by the virus as a receptor. It is difficult to assess the respective roles of NCAM and AchR, as receptor analysis has been performed in cells or tissues expressing both NCAM and AchR, such as N2a cells (33), IMR-32 cells (22), myotubes, mouse diaphragm cells, and neuromuscular junc- tions (14). In blocking experiments, NCAM ligands were as efficient, or even more efficient, in N2a cells expressing both NCAM and AchR as, or than, in BSR cells expressing NCAM only. If AchR is functionally active in N2a cells, this indicates
that there may be functional synergy or a close-contact relationship between the two molecules. However, evidence is accumulating that NCAM and AchR function independently both in vitro and in vivo. First, the capacity of RV to infect NCAM-transfected cells lacking AchR clearly demonstrates that NCAM allows RV entry independently of AchR. In vivo, RV infects cells that are AchR negative such as microglia, Schwann cells, and spindle cells and reaches the nervous system after inoculation into sites lacking AchR such as the footpad or the anterior chamber of the eye or by intranasal instillation. Different sites, especially the olfactory bulb, suggest that there may be functional synergy or a close-contact relationship, and promotes integration into primary cerebellum cell aggregates. Cell Adhesion. 2:287–298.


