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Expression and distribution of the receptors for coxsackievirus B3 during fetal development of the Balb/c mouse and of their brain cells in culture

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Abstract

This study was designed mainly to determine the relationships between the expression and distribution of the cellular receptor proteins for coxsackievirus B3 (CVB3) and susceptibility of mouse brain cells during fetal development of Balb/c mice. Immunoblot analysis of fetal extracts demonstrated that the CVB3 receptor proteins were first expressed at day 14 of the fetal stage, and that maximal expression of the cellular receptor occurred at near term or newborn stage. Results also suggested that newborn mouse brain tissue expressed much larger quantities of viral receptor proteins, compared to other tissues. In vitro studies showed that both mouse neurons and astrocytes could be infected by two CVB3 strains, pantropic CVB3 Nancy strain (CVB3N) and myocardiotropic CVB3 Woodruff strain (CVB3W). CVB3N, however, replicated and grew to high titer in primary astrocyte cultures and in primary neuron cultures, whereas, primary astrocyte cultures were relatively resistant to CVB3W. Virus binding assays revealed that CVB3N bound faster and in greater amounts to mouse brain cells than CVBW. These two virus strains, however, were found to share the same receptor specificity by virus competition assays. The number of virus binding sites for CVB3 on newborn mouse brain cells was approximately 1.8×10^4 per cell. The data suggested that preferential expression of the cellular receptors on newborn mouse brain cells may be related to their high susceptibilities to CVB3 infection. Copyright © 1996 Elsevier Science B.V.

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1. Introduction

CVB3, a member of the picornavirus family, causes a wide range of diseases in humans, includ-

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ing pancreatitis, hepatitis, meningitis, encephalomyelitis, myocarditis, myalgia, etc. (Woodruff, 1980; Melnick, 1990; Pavesi et al., 1992). Earlier studies revealed that the CVB frequently produced generalized infections, especially in the central nervous system (CNS) of the newborn, whereas they were responsible for relatively mild or subclinical infections in adults (Kunin, 1962; Iwasaki et al., 1985; Pavesi et al., 1992). In a mouse model, the brain is considered to be one of the most susceptible organs of newborn mice to infection by CVB3. Severe encephalitis and meningitis could be induced in the newborn regardless of the route of virus inoculation, but the susceptibility of the CNS to CVB3 infection decreased with age (Grodums and Dempster, 1959, 1961; Kunin and Halmagyi, 1961). Balb/c mice have been used widely to study the pathogenesis of CVB3 because these inbred mice are highly susceptible to CVB3 infection (Minnich and Ray, 1980; Grun et al., 1988).

Many studies have been carried out to identify the mechanisms of age related susceptibility to CVB infection (Grodums and Dempster, 1961; Kunin and Halmagyi, 1961; Kunin, 1962; Melnick, 1990). One likely mechanism is the diminution in the cellular receptors with age, since much evidence suggested that cellular receptors are major determinants of viral pathogenesis (Crowell et al., 1985; Crowell et al., 1986). It was found, for example, that virus binding activity of brain cells is the greatest in newborn or suckling mice and this activity diminishes with time. This observation correlated with a dramatic reduction in susceptibility of the CNS to CVB3 infection over the first 8 weeks of age (Kunin, 1962; Grodums and Dempster, 1961). In vitro studies also have shown that a rat skeletal L8 cell line was susceptible to CVB3 only when it was differentiated (fusion ready) (Schultz and Crowell, 1983). These data have suggested that the molecular basis of the age and tissue specific susceptibility may be related to expression of the cellular receptor proteins for CVB3.

Several CVB3 strains or variants have been identified and characterized. CVB3 Nancy (CVB3N), a prototype virus, could infect most mammalian tissues, especially in brains, while CVB3 Woodruff (CVB3W) that was originally isolated from a patient with myocarditis had a special tropism for the heart, causing extensive myocarditis in humans and mice (Woodruff, 1980; Grun et al., 1988). Further studies have indicated that CVB3W had two frame-shift mutations and multiple amino acid substitutions in VP1, VP2, VP3 as compared with CVB3N (Lindberg et al., 1987; our unpublished data). Another virus variant isolated by blind passage in rhabdomyosarcoma cells (RD cells) gained a second receptor specificity (HR2) (Reagan et al., 1984; Hsu et al., 1990). The phenotypic change of CVB3RD has been attributed to mutations in the P1 region of the genome (Lindberg et al., 1992).

Recently, we identified four receptor proteins (p46, p44, p36 and p33) on newborn mouse brain cells that were responsible for binding CVB3. These murine receptor proteins were immunologically different from those of human HeLa cells (HR1), since anti-mouse receptor antibodies did not recognize the human receptors, and vice versa (Mapoles et al., 1985; Xu et al., 1995). Interestingly, antibodies made against purified individual receptor proteins (anti-p46, p36 and p33) specifically recognized the four receptor proteins and blocked the binding of CVB3 to the murine cells. These antisera, which were absorbed with mouse L cells, did not react with any human, rat and other mouse cellular proteins. It appeared that all four receptor proteins were structurally related since these individual antibodies recognized all four receptor proteins. P46 was shown to be a sialic acid linked glycoprotein, but there was no evidence that low molecular weight forms of the receptor proteins were proteolytic products or deglycosylated forms of P46 and p44. The exact relationships among these four receptor proteins are still unknown. Although the receptor for CVB3RD has been identified as decaying accelerating factor (DAF) on human cells (Bergelson et al., 1995), the nature of the cellular receptor for other CVB3 strain (CVB3N and CVB3W) remains unclear. Whether the unique heart tropism of CVB3W reflects use of a receptor specificity different from that for CVB3N is unknown.

This investigation was designed to examine the expression and distribution of the cellular recep-

tor proteins of Balb/c fetal mouse brain tissue and extracts of other tissues during gestation and after birth. In addition, the interaction of the receptors on specific types of brain cells (neurons and astrocytes) in vitro with two CVB3 strains having different tissue tropism was studied. Brain cells were selected for use in this study, because they were expected to have significant amounts of receptors and mouse hearts were too difficult to evaluate in a comparative way due to technical problems of cell dissociation and to relatively few receptors per gram of heart tissues (McDermott and Morgan, 1987; Kunin, 1962). The study revealed a unique expression and distribution pattern of the cellular receptors of tissue extracts for CVB3 during animal development, which correlated well with reduced susceptibility of mice as they age.

2. Materials and methods

2.1. Animals, cell lines, viruses and reagents

Balb/c mice were purchased from Jackson Laboratory, and bred in the animal facility of Hahnemann University. All mice were tested to be free from mouse hepatitis virus and enteroviruses.

The human HeLa cells, BGM kidney cells and mouse L cells were maintained in monolayer in the minimal eagle medium (MEM) in Hanks balanced salt solution supplemented with 15% calf serum (Hsu and Crowell, 1989).

CVB3 Nancy, Poliovirus type 1, and labeling of viruses with [^{35}S]methionine were described previously (Reagan et al., 1984). CVB3 Woodruff strain originated from the late Dr. Jack F. Woodruff of Cornell University. The approximate number of virus particles per ml was estimated, based on 1 A260 unit was equivalent to 10^{13} particles (Hsu and Crowell, 1989) and the specific activity of ^{35}S-labeled CVB3 was calculated from the data obtained. Concentrated virus pools were prepared from BGM cells infected with virus for 9 h at 37°C (Mapoles et al., 1985).

Rabbit anti-glial fibrillary acidic protein antibody (anti-GFAP) was purchased from ICN Co. All other reagents, including mouse monoclonal anti-GFAP, monoclonal anti-neuronal filament proteins P120 (anti-NF), rabbit anti-NF, goat anti-mouse IgG conjugated with tetramethylrhodamine isothiocyanate (TRITC), goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC), Poly-D lysine and other chemical reagents, were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

2.2. Anti-mouse receptor antibodies

Preparation of mono-specific rat antibodies against mouse receptor proteins p46 and p36 (anti-p46 and anti-p36) were described previously (Xu et al., 1995). The immunized rats were boosted twice for an additional 3 months to generate higher titers of antibodies. Antibodies were incubated with receptor negative mouse L cells at a ratio of 1:1 (v/v) at 4°C overnight, to remove nonspecific antibodies. Anti-p46 and anti-p36 were used to detect the receptor proteins under the reducing and nonreducing conditions, respectively.

2.3. Preparation of solubilized fetal and newborn mouse tissues

Mouse fetuses were collected from pregnant mice, starting from day 13–20 post-coitus. The tissues were minced into small pieces, washed with cold phosphate buffered saline (PBS) twice and homogenized by a Dounce homogenizer in an ice-cold bath. Newborn mouse tissues (brain, heart, lung, liver, kidney, skeletal muscle and skin) were taken from the same animals and treated as above. Solubilization of the homogenized tissues with 1% Triton X-100 (TX100) was described previously (Xu et al., 1995).

2.4. Determination of protein concentration

The BCA protein assay was used to determine the amount of protein in the solubilized tissues at room temperature (RT). The standard was bovine serum albumin (BSA). All procedures followed the protocol provided by Pierce (Rockford, IL).

2.5. Analysis of the receptor proteins by virus-blot and immunoblot

Approximately 100 μg of the solubilized fetal and newborn mouse tissues were subjected to 10% SDS–PAGE under nonreducing or reducing conditions (Laemmli, 1970). After electro-transfer onto polyvinylidene difluoride (PVDF) membrane (BioRad), the receptor proteins were probed with 2×10^6 CPM of ^{35}S-labeled CVB3N (under non-denaturing and nonreducing conditions only). Detection of receptors by anti-receptor antibodies was described previously (Xu et al., 1995). Briefly, anti-p36 was used at a 1:1000 dilution (under nonreducing conditions) and anti-p46 at 1:2000 dilution (under reducing conditions), followed by incubation with 1:20 000 diluted rabbit anti-rat IgG conjugated with peroxidase. The reactions were visualized by autoradiography for virus-blots and enhanced chemiluminescence (ECL kit, Amersham, UK) for immunoblots. Molecular weight markers (rabbit muscle phosphorylase B, 97.4 kDa; bovine serum albumin, 69 kDa; chicken egg albumin, 46 kDa and bovine carbonic anhydrase, 30 kDa) were transferred onto PVDF membrane and stained with colloidal gold solution (Bio-Rad). [^{14}C]methylated molecular weight markers were used in autoradiography.

2.6. Preparation of dissociated newborn mouse brain cells

The method for dissociation of newborn mouse brain cells has been described previously (Dreyfus and Black, 1990). Briefly, the cortex was taken from the brains of less than 24-h-old newborn Balb/c mice. Meninges and blood vessels were removed as much as possible. The brain cells were minced into small pieces and dissociated by 10–20 strokes with a Pasteur pipette. The cell suspensions were passed through a # 500 mesh screen and residual cell debris was removed by centrifugation at $900 \times g$ for 5 min. Whole cells in the pellets were dispersed in the binding buffer (3% fetal calf serum (FCS)-20 mM HEPES-PBS, PH 7.4), and kept at 4° until used.

2.7. Primary astrocyte and neuron cultures

Primary astrocyte cultures were prepared according to the method of Dreyfus and Black, 1990. Briefly, dissociated brain cells were suspended in nutrient medium (MEM supplemented with 2 mM glutamine, 7.5% FCS, 6 mg/ml glucose, 5 mg/ml penicillin and streptomycin) and seeded into culture dishes or flasks. Following attachment of the cells, the medium was exchanged with growth medium (MEM supplemented with 2 mM glutamine, 15% FCS, 6 mg/ml glucose, 5 mg/ml penicillin and streptomycin). Cultures were incubated at 37°C in a humidified incubator with 5% CO_2 for two days. Loosely attached oligodendrocytes were removed by gently shaking overnight at 37°C. Over 90% of the cells in primary cultures were astrocytes as identified by indirect immunofluorescent test (IFT) using anti-GFAP antibody after 7 days of incubation.

Primary neuron cultures also were prepared according to Dreyfus and Black, 1990. Dissociated brain cells were washed and resuspended with serum-free medium (Ham's F-12 medium supplemented with 50% MEM, 2 mM glutamine, 6 mg/ml glucose, 5 mg/ml penicillin and streptomycin, 60 μM putrescine, 20 nM progesterone, 100 μg/ml human transferrin, 25 μg/ml bovine insulin, 30 nM selenium). Cell suspensions were plated into the dishes or flasks coated with poly-D lysine, and incubated as described above. The cultures consisted of more than 95% neurons, as confirmed by IFT using rabbit anti-NF protein antibodies.

2.8. Virus binding assay

Mechanically dissociated cells or trypsinized cells (1×10^6) were washed once with the binding buffer and incubated with [^{35}S]CVB3 in 600 μl of buffer at RT. Duplicate aliquots (50 μl) of cell-virus suspension were removed at intervals of 1, 5, 10, 20, 30, and 60 min. The cell pellets were washed three times with binding buffer, dissolved in 0.5 ml of 0.5 M NH_4OH, and assayed for radioactivity by liquid scintillation (Hsu and Crowell, 1989).

2.9. Determination of the number of binding sites for CVB3

Dissociated newborn mouse brain cells (5×10^5), prepared in triplicate, received 0.4 ml of ^{35}S-labeled viruses, respectively, diluted in the binding buffer and incubated at RT (rotating gently) for 60 min. After binding, cells were washed twice and assayed for radioactivity. The number of virus particles bound to cells was calculated from specific radioactivity (CPM/virus particle) associated with cells. The cell-associated label was used to calculate the number of virus particles bound to each cell, and plotted against the number of labeled particles added per cell. The approximate number of specific binding sites per cell was determined from the intercept of the slope of the nonspecific binding at zero input (Hsu and Crowell, 1989).

2.10. Determination of the types of brain cells susceptible to CVB3 infection using an indirect immunofluorescent test (IFT)

The methods were modified from previous reports (Dreyfus and Black, 1990; Lavi et al., 1987). Briefly, cells were grown in 85% confluent monolayers in slide culturing chambers (Lab-Tek) and challenged with approximately 50 PFU/cell of CVB3 for 24 h. The infected cells were fixed with acetone, and reacted with a 1:100 dilution of rabbit antiserum prepared against VP1 and VP3 of CVB3 (anti-VP1 and 3) (Beatrice et al., 1980) for 45 min at 4°C, followed by incubation with a 1:40 dilution of goat anti-rabbit IgG conjugated with FITC for 45 min at 4°C. Cell types were identified, respectively, by 1:200 dilution of the monoclonal anti-NF (for neuron cultures) or 1:200 dilution of the monoclonal anti-GFAP (for astrocyte cultures), and a 1:16 dilution of rabbit anti-mouse IgG conjugated with TRITC, according to the methods referred to above. Fluorescence was observed at a wavelength of 495 nM for FITC or at 540 nM for TRITC. Controls consisted of uninfected primary cell cultures (negative viral antigen control) and PBS mock treatment of cultures (antibody control).

2.11. Production of virus in primary cell cultures

Seven-day-old monolayers of primary cell cultures ($\sim 1 \times 10^6$ cells) were infected with about 1×10^6 PFU of CVB3N or CVB3W, and washed twice with PBS. To avoid washing off the weakly bound virus, primary cell cultures and L cells (negative control) seeded with CVB3W were washed gently only once. All cell cultures were incubated at 37°C, humidified to 80% and CO_2 was added to 5%. Virus samples were collected at intervals of 0, 2, 8, 16, 24, 48 and 72 h and frozen-thawed for three cycles to release cell bound viruses. Titration of virus was performed on BGM cells by PFU assays (Lavi et al., 1987; Crowell, 1966).

2.12. Virus competition assay

About 1×10^6 cells were pretreated with $\sim 2 \times 10^8$ PFU/0.5 ml of unlabeled virus for 8 h. The pretreated cells were incubated with [^{35}S]CVB3N or [^{35}S]CVB3W for another 2 h. The cells were washed and the cell pellets were collected by centrifugation and dissolved in 0.5 ml of 0.5 M NH_4OH and counted for cell associated radioactivity (Hsu and Crowell, 1989).

3. Results

3.1. Expression of the cellular receptor proteins for CVB3 during development of fetal Balb/c mice

Many studies have suggested that susceptibility to CVB3 in the mouse varied with its development (Grodums and Dempster, 1959; Kunin and Halmagyi, 1961). The first experiment was designed to examine the expression of the cellular receptor proteins for CVB3 during fetal development.

Solubilized fetal mouse tissues were analyzed by immunoblot using anti-p46 and anti-p36, two mono-specific rat antibodies against mouse receptor proteins p46 and p36 (Xu et al., 1995). These two antibodies were raised by immunizing rats with each of the purified receptor proteins respectively, and they were found to specifically recog-

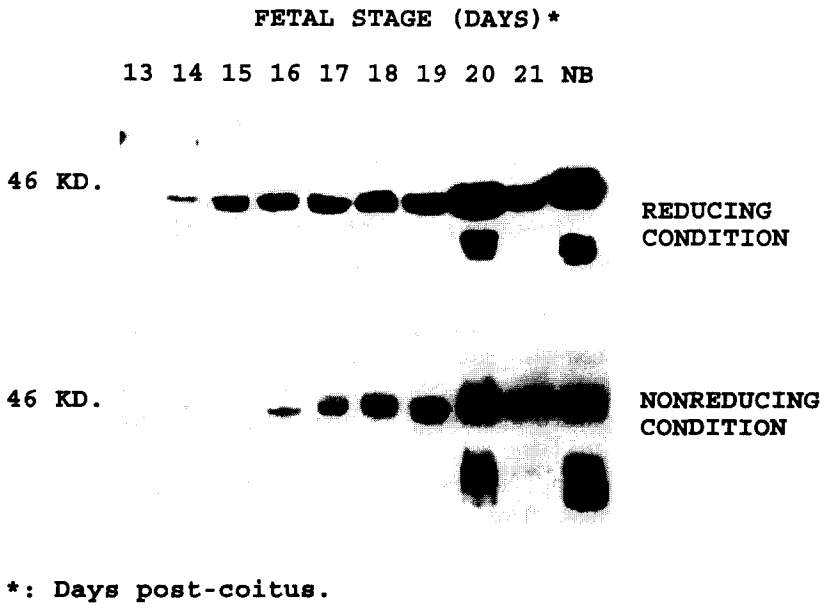

Fig. 1. Analysis of expression of CVB3 receptor proteins during fetal development of Balb/c mice by immunoblot assays. The receptor proteins in solubilized fetal tissues at daily intervals postcoitus, were separated by SDS–PAGE under reducing and nonreducing conditions, and transferred onto PVDF membrane for assay. Anti-p46 and anti-p36 antibodies were used to detect the reduced and nonreduced receptor proteins, respectively. Extracts of newborn mouse brain (NB) were used as positive control. Colloidal gold stained molecular weight markers are not shown (see Section 2).

nize each of the mouse receptor proteins on Western blots. Anti-p46 antibody worked better on Western blots under reducing conditions than nonreducing conditions, whereas, anti-p36 antibody only strongly recognized the mouse receptor proteins under nonreducing conditions (Xu et al., 1995). Therefore, anti-p46 and anti-p36 were used separately for detecting the mouse receptor proteins under reducing and nonreducing conditions, respectively. It was found that anti-p46 and anti-p36 detected these cellular proteins in fetal mice only after they had reached day 14 of gestation, under both reducing and nonreducing conditions, respectively (Fig. 1). The molecular size of the receptor proteins during the cellular development seemed not to change, however, two receptor protein bands with low molecular weight (p36 and p33) did not appear until day 20 of the fetus (see result under nonreducing conditions). Expression of the cellular proteins increased as development of the fetus advanced. The maximal expression of the cellular protein seemed to be at near term or the newborn stage. The data suggest that maximal expression of the receptor proteins at the newborn stage might be a major factor which contributes to the high susceptibility of neonatal mice to infection by CVB3.

3.2. Distribution of the cellular receptor for CVB3 in different tissues from newborn Balb/c mice

The finding that expression of the cellular receptor proteins is maximal near term or at birth of Balb/c mice suggested that separate tissues of these animals should be examined for the presence of receptor proteins, because it was known that not all of the mouse tissues at this stage were susceptible to CVB3 infection (Grodums and Dempster, 1959). To examine for the tissue distribution of cellular receptor proteins, different tissues were taken from the newborn mice and separately solubilized by a strong detergent, Triton X-100 (TX-100) (final concentration of TX-

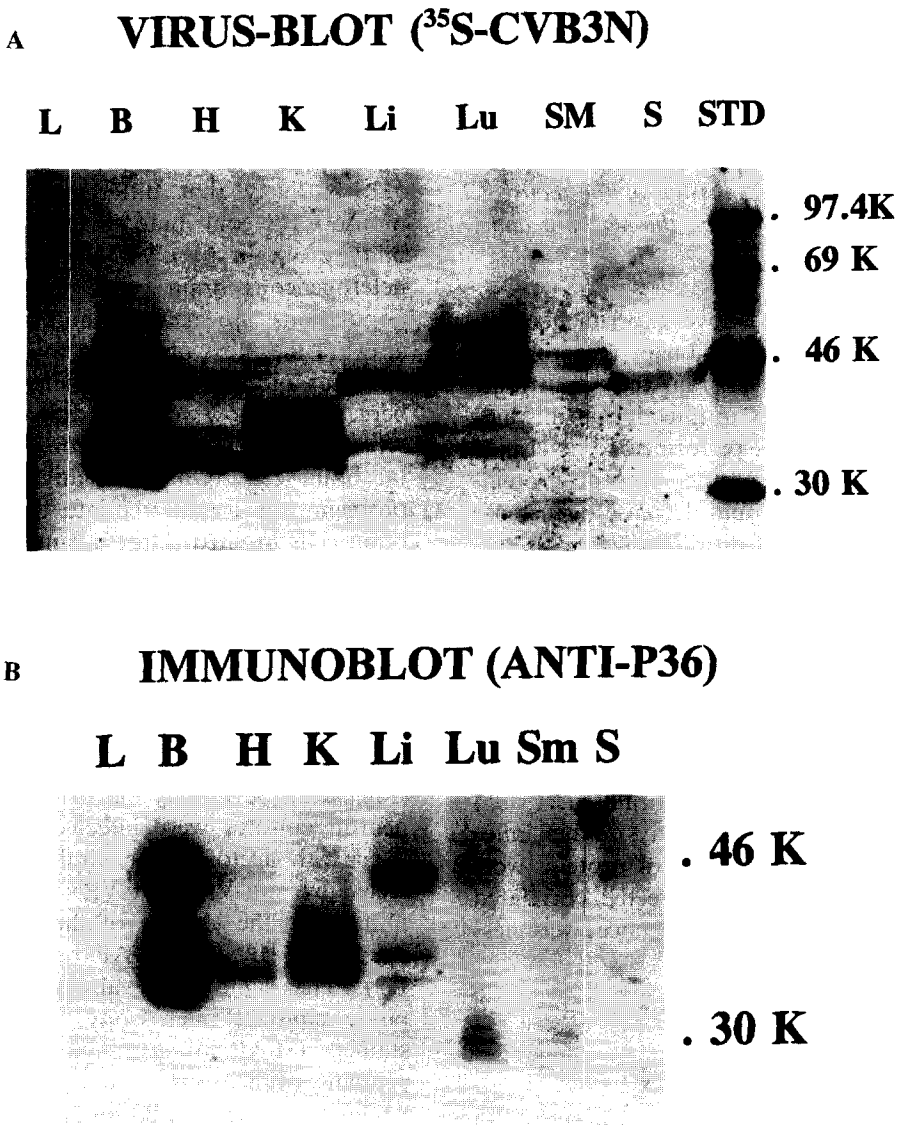

Fig. 2. Receptor proteins for CVB3 in different tissues of newborn Balb/c mice as detected in detergent extracts by virus-blots and immunoblots. The receptor proteins were separated by SDS-PAGE under nonreducing conditions, transferred onto PVDF membranes, and detected by ^{35}S-labeled CVB3N (A) and rat anti-p36 antibody (B). L, mouse L cells (negative control); B, brain; H, heart; Li, liver; Lu, lung; SM, skeletal muscles; S, skin; STD, ^{14}C-labeled protein standards (see Section 2).

100 1%) to maximize tissue solubilizing efficiency, especially for heart and skeletal muscle. The same amounts of the solubilized proteins from the different tissues were analyzed under nonreducing conditions by virus-blot using ^{35}S-labeled CVB3N (Fig. 2A) and by immunoblot using anti-p36 (Fig. 2B). Fig. 2 shows the distribution of the cellular receptor proteins for CVB3 among the different tissues. The brain expressed the largest quantity of each of four bands of CVB3 receptor proteins, whereas, heart and kidney expressed only two of them, p36 and p33. A small amount of receptor

proteins (p46 or p43) also was seen on lung and skeletal muscles. Liver appeared to have the same profile of expression of the receptor proteins as that of brain, although the quantity was much less.

3.3. Comparison of the expression of the mouse brain receptor proteins between the newborn and adult mice

The results from the above experiments suggested that abundant expression of receptors for CVB3 in newborn mouse brain may contribute to its higher susceptibility to infection at this age. The next experiment was performed to determine whether the expression of the receptor proteins for CVB3 in the brain would decrease or increase with mouse age. Mice at different ages were sacrificed and their brains assayed for receptor content as above. The results shown in Fig. 3 revealed that the maximal expression of the receptor proteins was found in newborns, although there was little difference between the expression of receptor proteins between newborns and 7 day old animals. However, the cellular receptor proteins for CVB3 were dramatically decreased as mice became older than 7 days. When mice were over 30 days old, the receptor proteins were almost undetectable, which directly correlated with earlier findings that older mice were less susceptible to CVB3 infection (Grodums and Dempster, 1961)

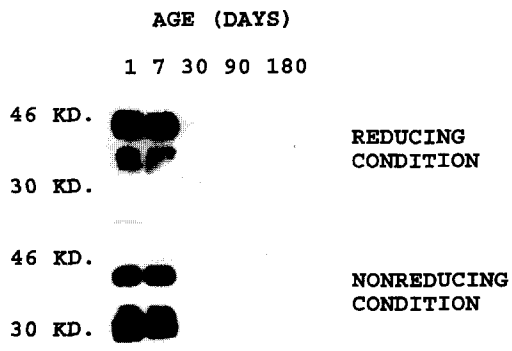

Fig. 3. Comparison of the expression of the brain receptor proteins for CVB3 in Balb/c mice at different ages. The processing of the brain extracts and detection of receptor proteins by antibodies (anti-p46 and anti-p36) were the same as in Fig. 1.

3.4. Determination of the major cell type of newborn mouse brain susceptible to CVB3

It is likely that different cell types in the mouse CNS contribute to the different susceptibility of brain to CVB3 infection. Among the heterogeneous brain cells, the majority of cell populations are neurons and astrocytes which also are the most important functional cell types (Dreyfus and Black, 1990; Kimelberg and Norenberg, 1989). However, it is difficult technically to separate astrocytes and neurons directly from total brain cells. One alternative to solving this problem was to differentiate them by culturing brain cells in vitro (Dreyfus and Black, 1990).

Primary astrocyte and neuron cultures were prepared from the cerebrum of the newborn mouse to identify the susceptible cell types (Fig. 4). There were more than 85–90% astrocytes and 95% neurons in primary astrocyte and neuron cultures, respectively, as confirmed by IFT using anti-GFAP (Fig. 4A2) and anti-NF proteins (Fig. 4C2). Seven day old primary astrocyte and neuron cultures were infected with CVB3, and fixed with acetone. The viral antigens were detected by anti-VP1 and VP3 antibodies (B2 and D2), while the identity of the infected cells was determined by anti-GFAP or anti-NF (B3 and D3). The same fields also were observed by phase contrast microscopy (B1 and D1). The stained GFAP of astrocyte or neurofilament proteins of neurons produced red fluorescence, while the stained CVB3 antigens (VP1 and VP3) showed the green-yellow color. The data demonstrated that both neurons and astrocytes could be infected by CVB3 in vitro. The neurons appeared to be more susceptible than astrocytes because almost 100% cells were destroyed by virus at 24 h post-infection (p.i.), whereas, only 50% of astrocytes were destroyed under the same conditions. Both astrocytes and neurons also were susceptible to CVB3W as well, however, the percentage of the infected cells was much less (50%) as compared with CVB3N (80–100%) (data not shown).

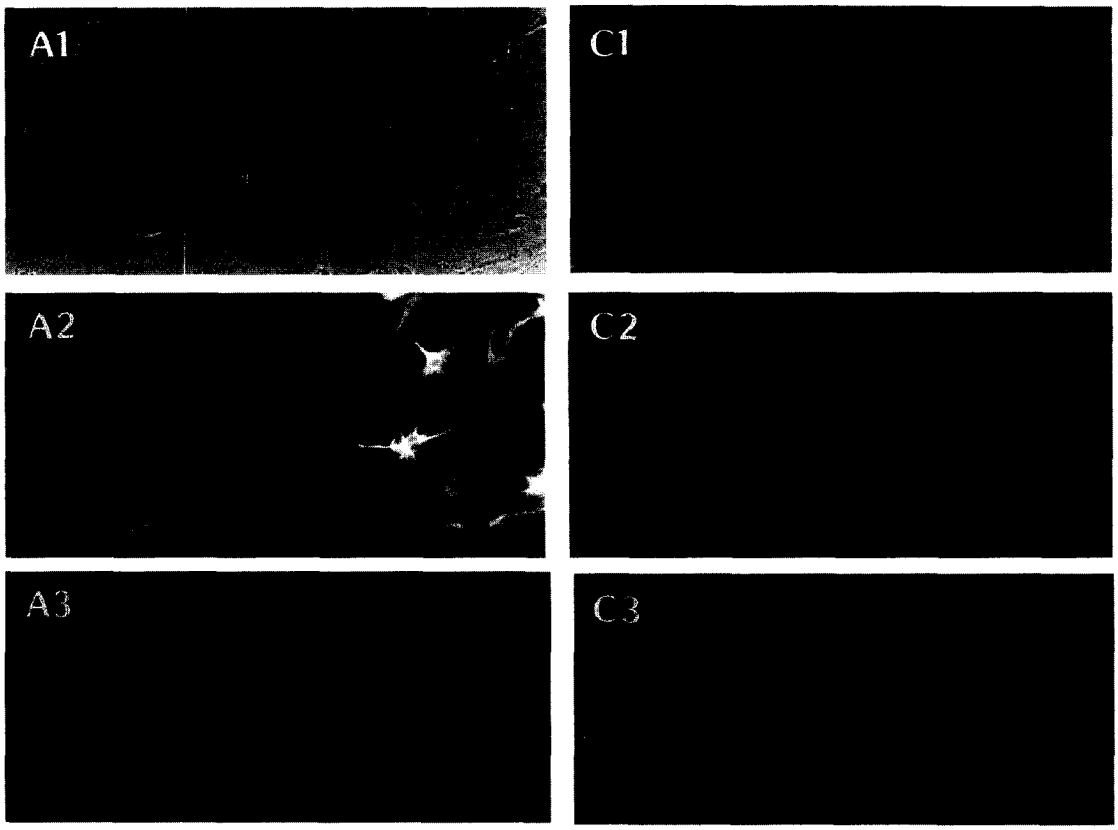

Fig. 4.

3.5. Replication of CVB3N and CVB3W in mouse brain cells

To confirm whether there was any difference between CVB3N and CVB3W replication in cultured brain cells as suggested by IFT, the production of CVB3N and CVB3W in primary neuron and astrocyte cultures, respectively was determined (Fig. 5). Fig. 5B indicates that growth of CVB3W in primary neuron cultures was slightly lower (20-fold) than that of CVB3N. Furthermore, CVB3W yields were significantly lower (1000-fold) in primary astrocytes than CVB3N, despite the higher initial input amount of CVB3W (Fig. 5A). These findings were not due to the insusceptibility of astrocytes to CVB3W because after prolonged incubation (72 h), astrocyte cultures were totally destroyed. In contrast, both CVB3W and CVB3N replicated equally well in HeLa cells, which was consistent with the fact that both strains use the same receptor on HeLa cells (unpublished data).

3.6. Comparison of binding activity of CVB3N and CVB3W to newborn mouse brain cells

The next experiment was performed to determine whether the difference in yields between CVB3N and CVB3W from mouse brain cells was related to different receptor binding activities between the two strains of CVB3. The binding kinetics of these two strains of virus to whole dissociated newborn mouse brain cells (Fig. 6A), primary astrocyte cultures (Fig. 6B) and primary neuron cultures (Fig. 6C) were examined. Each cell population of 1×10^6 cells received approxi-

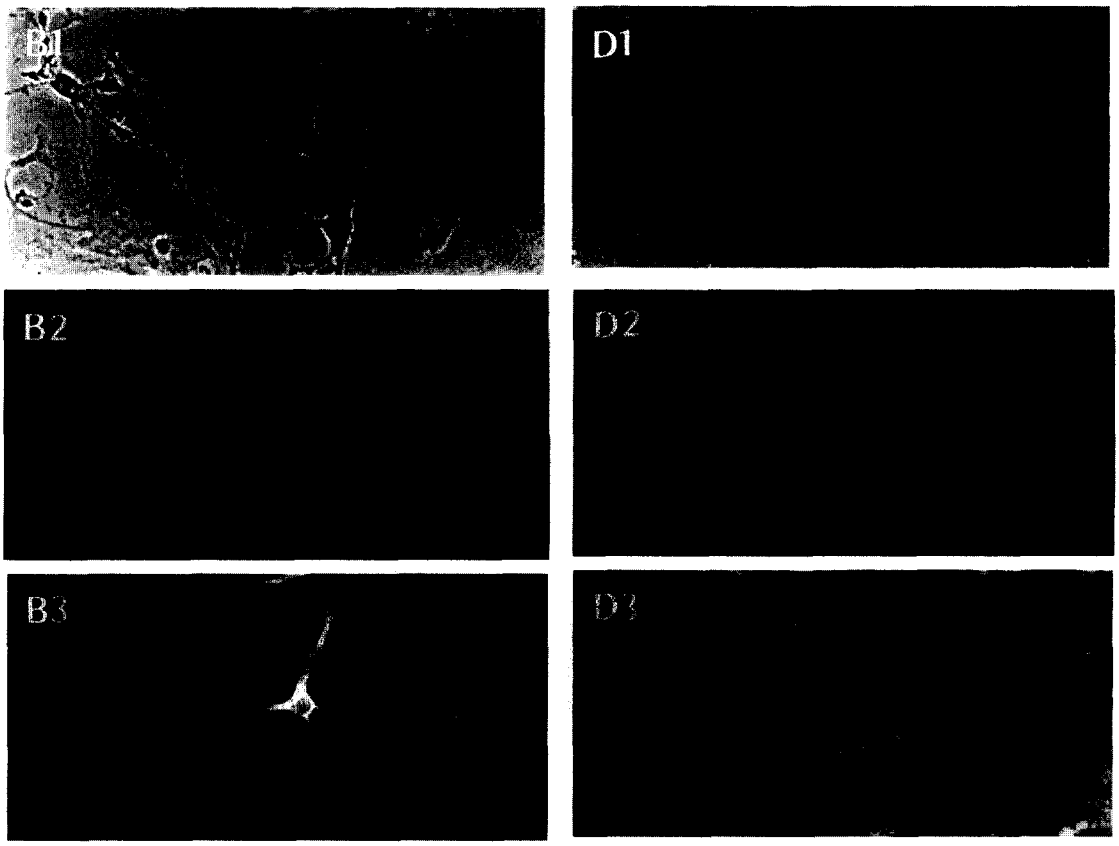

Fig. 4 Identification of the susceptibility of mouse astrocytes and neurons to CVB3 infection by indirect immunofluorescent assays in vitro. A1 and C1, normal seven day old primary astrocyte and neuron cultures under phase contrast microscopy, respectively; A2 and C2, normal primary astrocyte and neuron cultures examined by anti-GFAP or anti-NF proteins and goat anti-rabbit IgG conjugated with FITC, respectively; A3 and C3, uninfected primary astrocyte and neuron cultures examined by rabbit anti-CVB3 VP1 and VP3 antiserum, followed by incubation with goat anti-rabbit IgG conjugated with FITC (samples from the same cultures as in A1, or C1); B1 and D1, fixed primary astrocyte and neuron cultures infected with CVB3N under phase contrast microscopy, respectively; B2 and D2, detection of viral antigens in the infected cells by anti-VP1 and VP3 antiserum and FITC-conjugated goat anti-rabbit IgG (green-yellow); B3 and D3, astrocytic and neuronal identities of the infected cells revealed by monoclonal anti-GFAP or monoclonal anti-NF proteins and TRITC-conjugated rabbit anti-mouse IgG (red), respectively.

mately 1×10^5 CPM of ^{35}S-labeled viruses and the amount of virus label bound to the cell surface at intervals was determined. Fig. 6 shows that CVB3N bound to dissociated newborn mouse brain cells, astrocytes and neurons much faster and in greater amount than did CVB3W. This binding activity of virus was not sensitive to treatment with a mixture of trypsin-collagenase at RT (data not shown).

3.7. The receptor specificity of CVB3N and CVB3W on newborn mouse brain cells

Virus competition assays were performed to determine whether the difference between CVB3N and CVB3W binding to newborn mouse brain cells reflected a difference in receptor specificity between these two viruses. Mechanically dissociated newborn mouse brain cells and trypsinized HeLa cells were pretreated with large amounts of

non-labeled viruses at RT for 60 min, followed by addition of ^{35}S-labeled CVB3W (Fig. 7A) or CVB3N (Fig. 7B). The amount of cells associated virus label was determined by scintillation counter. The results shown in Fig. 7 indicated that CVB3N and CVB3W competed for the same receptors, suggesting that they share the same receptor specificity on newborn mouse cells.

3.8. Determination of the number of binding sites for CVB3 on newborn mouse brain cells

A virus saturation assay was used to analyze quantitatively the cellular receptors for CVB3 on the surface of newborn mouse cells (Hsu and Crowell, 1989). Serially diluted ^{35}S-labeled viruses were incubated with 5×10^5 cells, and the amount of bound virus was plotted against the amount of input virus. The approximate number of binding sites (or viral receptors) per cell was expressed as the intercept of the slope of the nonspecific binding at zero input. Fig. 8 shows a representative of

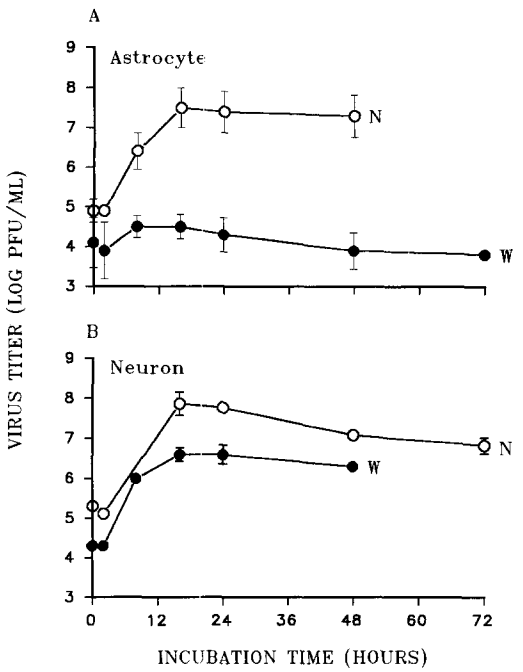

Fig. 5. Production of CVB3N and CVB3W in primary astrocyte (A) and neuron cultures (B). Virus titer was determined by PFU assays on BGM cells at the intervals indicated. (○), titer of CVB3N (N); (●), titer of CVB3W (W).

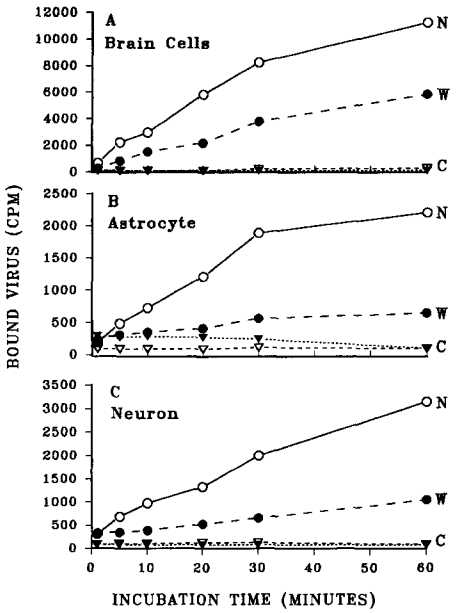

Fig. 6. Comparison of the binding activity of CVB3N and CVB3W to newborn mouse brain cells. One million dissociated newborn mouse brain cells (A), astrocytes (B) and neurons (C) were incubated with 1×10^5 CPM of ^{35}S-labeled CVB3N or CVB3W at RT, respectively. Mouse L cells were used as a negative control. (——○——), CVB3N (N) bound to brain cells; (— —●— —), CVB3W (W) bound to brain cells; (- - -▼- - -), CVB3N bound to L cells (C); (- - -▽- - -), CVB3W bound to L cells (C).

results obtained from three separate experiments. The mean of the virus binding sites or receptors for CVB3 on newborn mouse brain cells was 1.8×10^4 per cell. This number was close to that found for HeLa cells ($\sim 2 \times 10^4$ per cell) (Hsu et al., 1988), but is 40-fold more than that of mouse Yac-1 cells (Hsu and Crowell, 1989).

4. Discussion

A number of studies have indicated that the newborn mouse is more susceptible than the adult mouse to the group B coxsackieviruses (Grodums and Dempster, 1959; Kunin and Halmagyi, 1961; Melnick, 1990). However, there was no information regarding the onset of virus susceptibility of the mouse, in utero. Therefore, the expression of CVB receptor proteins during fetal development

was studied by immunoblots using anti-mouse receptor antibodies (Xu et al., 1995), since the development of cellular receptors is prerequisite to the development of cell susceptibility to infection (Crowell et al., 1985). The results showed that the earliest detectable expression of the CVB3 cellular proteins was at day 14 of gestation, with amounts increasing until birth of the mouse, at which time maximal expression was observed. These findings coincided with the high level of susceptibility of the newborn mouse to CVB infection. In addition, newborn babies also are considered to be in danger from CVB infection (Woodruff, 1980; Iwasaki et al., 1985; Pavesi et al., 1992).

Infection of newborn mice with the CVB results in a wide range of tissue damage, especially in the CNS (Grodums and Dempster, 1961). It has been shown that the CNS becomes infected prior to heart tissue of the newborn mouse, which could be a reflection of cellular receptor development (Grun et al., 1988). Consequently, we examined multiple tissues for the expression of CVB receptors from newborn mice. Immunoblot analysis of receptor proteins in detergent extracts of different tissues revealed that these proteins were expressed in a wide array of tissues, which coincided with the known wide tissue tropism of the CVB. It was found that the newborn mouse brain and liver had significantly more receptor proteins, than the other tissues examined. In addition, it was found that heart tissue expressed only p33 and/or p36, whereas, lung and kidney expressed p46 and/or p43. Since these receptor proteins of different size are antigenically related (Xu et al., 1995), the significance of finding different sized receptor proteins remains unknown (Koike et al., 1990) and must await the successful cloning of the receptor gene for resolution.

The notable neurotropism of CVB3 in newborn mice, in contrast to the relatively resistant adult mice, correlated with the relatively high amounts of CVB receptor proteins in the newborn mouse

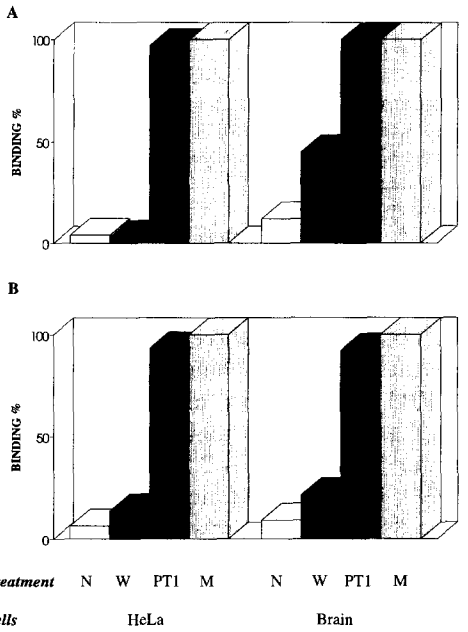

Fig. 7. CVB3N and CVB3W share the same receptor specificity on newborn mouse brain cells. Dissociated newborn mouse brain cells were pretreated with high titer of unlabeled viruses (N, CVB3N; W, CVB3W; PTI, Poliovirus type 1) at RT for 8 h. ^{35}S-labeled CVB3W (panel A) or CVB3N (panel B) were added, respectively, to the cells pretreated with unlabeled virus, at RT for additional 2 h. Binding of CVB3 to mock treated cells (M) was defined as 100%. Poliovirus type 1 (PTI) was used as a negative control. The results shown are the averages of three independent experiments.

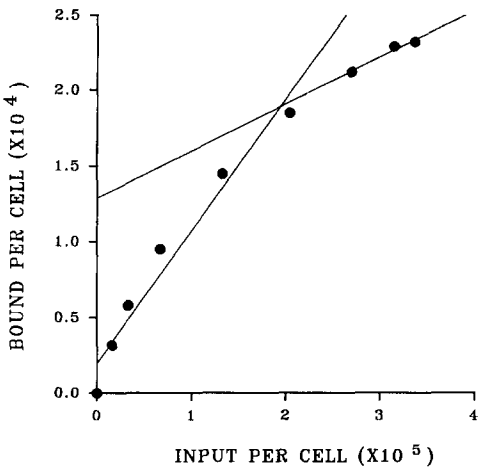

Fig. 8. Determination of the number of receptors for CVB3 on dissociated newborn mouse brain cells. Serial dilutions of ^{35}S-labeled CVB3N were incubated, respectively, with 1×10^6 dissociated brain cells at RT for 1 h. Virus particles specifically bound to the cells were counted for radioactivity. The amount of cell-associated labeled virus per cell was plotted against the amount of labeled input virus per cell.

brain. The results of our studies extended those of Kunin and Halmagyi (1961) in two ways: (1) four separate receptor proteins were monitored by immunoblot assays with receptor specific antibodies, and (2) detergent extracts of tissues were more sensitive to receptor assay (Krah and Crowell, 1982) than minced brain tissues. The net result, however, was to confirm that the amount of receptor proteins correlated directly with the reduced susceptibility of mice to CVB infection as the mouse ages (Kunin and Halmagyi, 1961).

The results of our study also identified two major cell types from the newborn mouse brain (neurons and astrocytes), which were major targets for infection by both virus variants (CVB3N and CVB3W) of CVB3 that were tested. Cultures of neurons and astrocytes, prepared from newborn or near term mice, were demonstrated to be susceptible to infection. The cell types were identified by protein specific monoclonal antibodies and virus replication was determined by virus specific antibodies in IFT. Even though the astrocytes were more susceptible to CVB3N and this virus attached more rapidly, than CVB3W, it was shown by virus competition assays that both viruses had the same receptor specificity. Thus, differing rates of virus attachment should not be taken as evidence that there are more receptors for the more rapidly binding virus (Crowell et al., 1985). Although CVB3W was less efficient to block binding of CVB3N and CVB3W to the receptors, blocking was accomplished by allowing more time for CVB3W to interact with receptors (data not shown). Low binding affinity of CVB3W compared with CVB3N may reflect differing mutations in VP1, VP2, VP3 regions of the CVB3W capsid. The data suggested that mutations in VP1, VP2 and VP3 regions of CVB3W only had minimal effect on the viral tropism. In a mouse model, CVB3W produces extensive myocarditis, whereas, CVB3N does not. However, whether the capsid proteins of the two CVB3 variants account for their different types of pathogenesis in mice is unknown. Further studies to identify the differences between the pathogenesis of these virus variants are needed, although the differences in neurovirulence between vaccine strains and wild type polioviruses have been found to be encoded in a conserved domain in the central part of the 5′-nontranslated region of the viral genome (Almond, 1987). A similar finding has recently been shown for the cardiovirulence of myocardiotropic CVB3 strains (Chapman et al., 1995). Further studies of the role of the capsid structure of virus variants by construction of chimeric viruses (Colonno et al., 1988; Lindberg et al., 1992) are needed to relate structure-function relationships between specific receptors and viral pathogenesis.

The results of this study also provide useful information for future attempts to purify receptor proteins in order to determine a partial amino acid sequence for cloning of the receptor gene from mice. Thus, the newborn mouse would be expected to have the highest amounts of receptor proteins and a correspondingly high level of receptor specific mRNA for preparation of a cDNA library for cloning of the receptor gene. The mouse serves as an excellent model of human infection by the CVB, and cloning of the murine receptor gene is a most desired project to determine the role of these receptors in the tissue tropism of virus variants in the pathogenesis of infection.

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