CRR in breath test. As shown in Figure 3, the phenylalanine breath test exhibited continuous levels of CRR in PAH deficiency from 0 (classical PKU) to 2.74% (mild HPA). These results are in agreement with various clinical manifestations of PKU. The CRR (*in vivo* PAH activity) could distinguish control subjects (15.4 ± 1.5%; range: 13.79–17.44%); the heterozygotes (10.3 ± 1.0%; 9.00–11.42%); and mild HPA (2.74%), mild PKU (1.13 ± 0.14%; 1.01–1.40%), and classical PKU (0.29 ± 0.14%; 0–0.93%) patients (Fig. 3). We found consistency between the CRR and clinical phenotype in the Japanese patients who were tested in this study. Patients with compound heterozygotes of mild (L52S, R241C, R408Q) and severe mutations had CRR of 1.0–1.4% and mild PKU phenotype, whereas patients with severe mutations for both alleles had CRR of <0.93% and classical PKU phenotype. In this study, we did not find inconsistency among clinical phenotype, CRR, and genotype.

BH_4 loading increased CRR from 1.13 ± 0.14 to 2.95 ± 1.14% (2.6-fold) in all four patients with mild PKU and also increased it from 2.74 to 7.22% (2.6-fold) in mild HPA patients. All patients with mild PKU and mild HPA in this study responded to BH_4. Two patients with classical PKU showed no increase in the CRR after BH_4 loading. BH_4-induced activation was proportional to residual PAH activity. In PTPS patients 1 and 2, serum phenylalanine was effectively controlled to ≤0.12 mM after administration of BH_4 at 3.4 and 6 mg · kg^{-1} · d^{-1}, respectively, with regular food. The CRR values in these two patients were 5.88 and 19.0%, respectively.

Correlation between CRR and phenylalanine levels without dietary treatment. Correlation between CRR and serum phenylalanine levels without dietary treatment was examined in 26 patients. Serum phenylalanine levels of four control subjects, four heterozygotes, and two patients with PTPS deficiency were measured before administration of phenylalanine in the breath test. Plasma phenylalanine levels of 16 patients with PAH deficiency were examined before phenylalanine-restriction therapy. As shown in Figure 4, CRR correlated inversely with phenylalanine concentration ($1/y = 0.69 + 1.02\ x$; $p < 0.0001$). This result indicates that phenylalanine levels can decrease steeply with a slight increase of CRR (from 0% or near 0% to 1–2%), and the clinical phenotype changes from classical PKU to mild HPA.

DISCUSSION

In the phenylalanine breath test, the administered ^{13}C-phenylalanine is absorbed in the intestine and transported to the liver cells through the portal vein. In the liver, ^{13}C-phenylalanine is converted to ^{13}C-tyrosine by PAH, then to homogentisic acid by tyrosine aminotransferase and dioxygenase, and is finally exhaled as $^{13}CO_2$. Thus, this test not only simply measures PAH activity but also evaluates the overall state of phenylalanine metabolism in humans, *i.e.* phenylalanine oxidation capacity. This test is expected to reflect the clinical phenotype of PKU. The phenylalanine breath test used in the present study does not require blood sampling or special

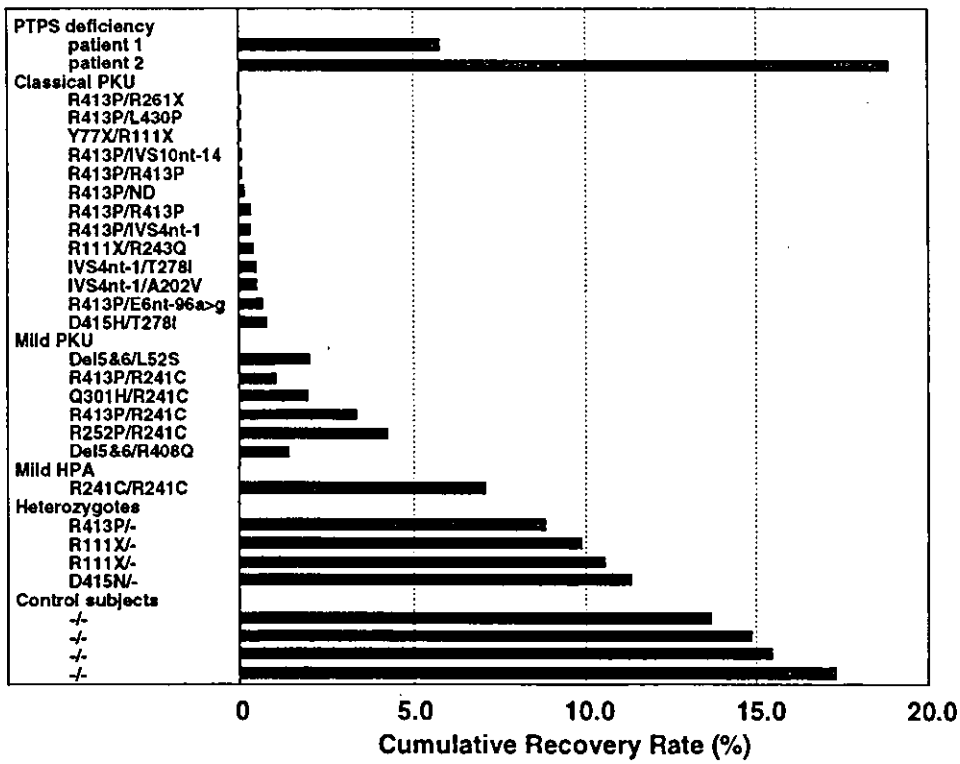

Figure 3. Phenylalanine oxidation capacity in control subjects, heterozygotes, patients with PAH deficiency, and patients with PTPS deficiency. ▨, CRR (%) values determined during 120 min after the ingestion of ^{13}C-phenylalanine without BH_4 dosing; ■, CRR (%) values determined during 120 min after the ingestion of ^{13}C-phenylalanine with BH_4 dosing. The detected mutations in PAH-deficient patients and heterozygotes are indicated in the left panel. -, no mutation; ND, not determined.

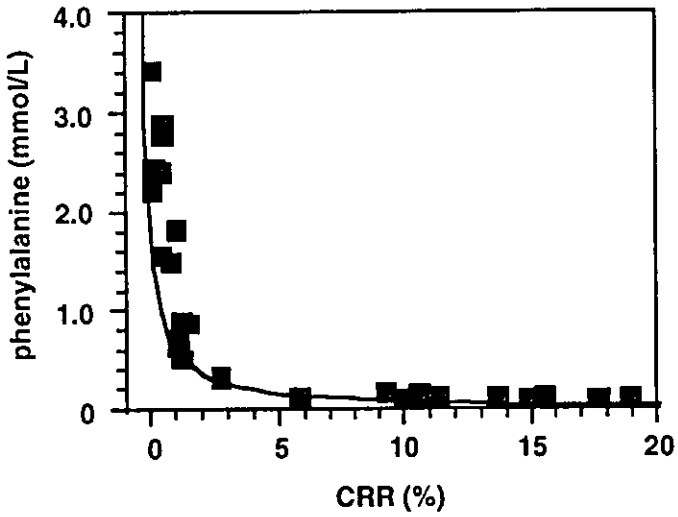

Figure 4. Correlation between pretreatment phenylalanine levels and CRR in control subjects, heterozygotes, patients with PAH deficiency, and patients with PTPS deficiency (n = 26).

conditions such as high serum phenylalanine levels. The results are deduced from analysis of the expired gas collected over a short period of time. Thus, the phenylalanine breath test is simple and noninvasive and can be repeated several times.

The CRR in phenylalanine breath test showed a continuum from classical PKU to mild HPA (Fig. 3). This finding is in agreement with the clinical notion that PKU is a highly heterogeneous disease for the clinical phenotype, which is caused by the strong heterogeneity of PKU mutations. In fact, 18 different mutations were detected in 20 patients in the present study. Patients with classical PKU, mild PKU, and mild HPA had significantly different CRR values of <1%, 1–1.4%, 2.4%, respectively. Therefore, we can predict the clinical phenotype from the CRR. Furthermore, CRR can be used to determine adherence to diet therapy. Concerning the effects of BH_4 on PAH, all five patients with mild PKU and mild HPA responded to BH_4 loading and showed an increase in CRR, which was proportional to the residual PAH activity after BH_4 dosing. In patients with classical PKU, BH_4 loading did not increase CRR. When the CRR is 1% or higher before BH_4 dosing, the response to BH_4 may be expected (Fig. 3). A CRR value of 5.88% in patients with PTPS deficiency indicated <0.12 mM of serum phenylalanine levels on long-term BH_4 therapy. These findings suggest that the cumulative recovery of 5 to 6% is also sufficient for maintaining serum phenylalanine level (0.12 mM) in patients with PAH deficiency and that our test may be potentially useful for determining the optimal dosage of BH_4 for long-term medication of BH_4-responsive HPA patients.

The frequency and types of PKU mutations differ greatly between whites and East Asians (21,24). Mutations associated with mild phenotype and BH_4-responsive PAH deficiency in East Asians also differ from those in whites. In the present study, all patients with mild PKU phenotype were compound heterozygotes with severe and mild mutations, which included R241C (*in vitro* PAH activity; 25%) (25), L52S (27%) (22), and R408Q (55%) (26), as shown in Table 1. Mild HPA was R241C homozygote. The patient with R408Q (*in vitro* PAH activity, 55%) and Del 5&6 had higher CRR than four patients with R241C (*in vitro* PAH activity, 25%) and severe mutations (R413P, Q301H, and R252P). The phenylalanine oxidation capacity, *i.e.* CRR, determined by our test stands between the clinical phenotype and the genotype and links them together. In the present study of Japanese patients, none of the patients showed any disagreement among clinical phenotype, phenylalanine oxidation capacity, and genotype. It follows that the genotype determined by both alleles mainly specifies PAH activity, which in turn specifies the clinical manifestations in an individual.

However, in East Asians, discordance between BH_4 respon siveness and genotype has been reported (12). Two patients with P407S mutation were described, one as a nonresponder (P407S/R111X) and the other as a responder (P407S/R252W) to BH_4. This different responsiveness is thought to be due to another mutation in each patient (R111X: stop codon mutation; R252W: missense mutation) on PAH protein synthesis. In whites, inconsistencies associated with BH_4 responsiveness are reported concerning Y414C, L48S, I65V, and R261Q mutations, and the BH_4 responders and nonresponders are present i individuals with the same mild mutation (17).

The cause for discordance among clinical phenotype including BH_4-responsive PAH deficiency and genotype is not ye clear. In addition, the mechanism responsible for the recover of defective PAH activity after BH_4 loading remains elusive Two broad factors determine the effect of BH_4 on PAH: *1*) BH site: absorption, distribution, and metabolism of orally admin istered BH_4, and 2) PAH site: interaction between BH_4 an PAH gene and protein. Concerning BH_4 site, absorption o BH_4 is minimal and unstable and differs greatly from on individual to another (Suntory Co., personal communication) The optimal dose and the duration of BH_4 administration fo the diagnosis of BH_4-responsive PAH deficiency remain un known. Bernegger *et al.* (27) pointed out that a single dose o 20 mg/kg of 6R-BH_4, the active form, was 5–20 times mo effective than smaller doses of 6R- BH_4 or 6R, S-BH_4 an induced a response in 70% of patients with mild PAH defi ciency. With regard to the optimum BH_4 dose for long-tei control of patients with BH_4-responsive PAH deficiency, fa vorable blood phenylalanine levels were obtained at a BH dose of 5–10 mg/kg. It may be necessary to repeat BH_4 dose over several days for unstable absorption of BH_4. In our stud using BH_4 at 10 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} for 3 d, a rise of PAH activit was noted in all patients with mild PKU and mild HPA.

Concerning PAH site factors, various mutations associate with BH_4 responsiveness have been identified, and some mu tations were outside the catalytic domain or the locus associated Km variant for BH_4 of the PAH enzyme. Dire effects of BH_4 are suspected. In other words, BH_4 may u regulate the expression of the PAH gene, stabilize PA mRNA, and facilitate and stabilize the formation of function PAH tetramers (16). Figure 4 provided in this study seems t confirm the proposal put forward by Scriver (28): "gene dosag effect in PAH deficiency." Figure 4 may clarify the causes an the mechanisms of the BH_4 responsiveness in mild PA deficiency and the discordance between genotype and clinic·

phenotype. Mutations that were identified in cases with discordance between genotype and clinical phenotype were basically related to the mild genotype. Patients with the mild clinical phenotype exist at the turning point of the correlation curve shown in Figure 4. This mild phenotype is produced by a small residual PAH activity, which is specified to the genotype. The formula suggests that a slight increase of the CRR at the turning point by certain effectors should greatly reduce blood phenylalanine level and cause transformation to a mild phenotype. In contrast, a slight decrease of the CRR leads to a rise in blood phenylalanine level and subsequently leads to transformation to a severe phenotype. Patients with mild mutation could become milder or more severe by certain effectors of the PAH enzyme. BH_4 is advocated as a strong effector to influence mutations at the turning point.

The phenylalanine breath test is useful for the diagnosis of BH_4-responsive PAH deficiency and determination of the optimal dosage of BH_4 without increasing blood phenylalanine level. To clarify the discordance between clinical phenotype including BH_4 responsiveness and genotype, it is important to investigate both genotype and phenylalanine oxidation capacity and to further accumulate such data.

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Infection of Different Cell Lines of Neural Origin with Subacute Sclerosing Panencephalitis (SSPE) Virus

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Abstract: **Measles virus is the causative agent of subacute sclerosing panencephalitis (SSPE). The viruses isolated from brain cells of patients with SSPE (called SSPE viruses) are defective in cell-free virus production *in vitro*. To investigate the cell tropism of three strains of SSPE virus (Osaka-1, Osaka-2, Osaka-3), SSPE virus-infected cell cultures were treated with cytochalasin D to prepare virus-like particles (CD-VLPs). All CD-VLPs formed syncytia after infection in CHO cells expressing CD150 but not in those expressing CD46. In addition, an antibody to CD46 did not block the infection of Vero cells by SSPE CD-VLPs. The results were consistent with our previous suggestion that one or more unidentified receptors might be involved in the entry process. Infection with the CD-VLPs from three SSPE strains was further examined in different human cell lines, including those of neural origin, and was found to induce syncytia in epithelial cells (HeLa and 293T) as well as neuroblastoma cells (IMR-32 and SK-N-SH) with varying efficiency. SSPE CD-VLPs also infected glioblastoma cells (A172) and astrocytoma cells (U-251) but syncytial formation was rarely induced. These epithelial and neural cell lines were not permissive for the replication of wild-type MV. Together with our previous observations, these results suggest that the cell entry receptor is the major factor determining the cell tropism of SSPE viruses. Further studies are necessary to identify other viral and/or cellular factors that might be involved in the replication of SSPE virus in specific neural cells and in the brain.**

Key words: **Measles virus, Syncytium formation, Receptor, Cytochalasin D**

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative disease caused by persistent infection of the central nervous system (CNS) with measles virus (MV). SSPE viruses, variant MVs isolated from the brains of patients with SSPE, differ biologically and genetically from MVs isolated from patients with acute measles. When the nucleotide sequences of the genome of SSPE virus strains were compared to the consensus sequences of MV, many mutations were found, particularly in the entire M gene and in part of the F gene (2, 4, 6, 9–13, 33, 47, 59). We have characterized three strains of SSPE virus (designated Osaka-1, Osaka-2, and Osaka-3) (44), compared them with their possible progenitor MV strains, and confirmed some essential SSPE-associated alterations (3, 5, 42, 50). We have also demonstrated the occurrence of SSPE virus-induced acute encephalopathy in hamsters and carefully monitored nucleotide sequence alterations during the course of experiments (29, unpublished observation).

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Abbreviations: CD-VLPs, virus-like particles prepared by treating infected cells with cytochalasin D; CHO, Chinese hamster ovary; CNS, central nervous system; CPEs, cytopathic effects; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; IFN, interferon; MV, measles virus; PFU, plaque-forming units; SSPE, subacute sclerosing panencephalitis; VSV, vesicular stomatitis virus.

SSPE viruses cause neurological disorders in experimental animals (such as hamsters and mice) when inoculated into the brain. Wild-type MVs as well as vaccine or laboratory strains do not usually cause neurological symptoms in genetically unmodified adult hamsters and mice. The molecular mechanisms underlying neurovirulence are not well understood. The involvement of host cell surface receptors for MV in neurovirulence is one of the important factors (48). Two MV receptors have been identified: CD46 (membrane cofactor protein for complement) (17, 40) and CD150 (signaling lymphocyte activation molecule, SLAM) (20, 27, 57).

When compared to MVs isolated from patients with acute measles, SSPE viruses are little known in many respects. Kobune et al. (32) reported that susceptibility of B95a cells (a marmoset B lymphoid cell line transformed by Epstein-Barr virus) to MV in clinical specimens is 10,000 times that of Vero cells. On the other hand, SSPE viruses have generally been isolated from cells of non-lymphoid origin, such as Vero (16, 25, 35, 37), BSC-1 (7), and primary human embryonic lung cells (58). We have tried to isolate our three SSPE viruses by cocultivation of brain cells from patients with SSPE with three different cell lines, Vero, B95a, and primary human embryonic lung cells (44). Although two of the three SSPE strains could be isolated by cocultivation with B95a cells, all strains were most successfully isolated by cocultivation with Vero cells. Some reports demonstrate that adaptation to Vero cells is accompanied by specific amino acid replacements within the H protein (Asn to Tyr at position 481 or Ser to Gly at position 546) (28, 46, 52, 60). However, such amino acid replacements were not found in the H proteins from our SSPE viruses isolated and passaged repeatedly in Vero cells (22). Our recent study of the receptor usage of SSPE viruses, using the vesicular stomatitis virus (VSV) pseudotype system, revealed that SSPE viruses can use SLAM, but not CD46, for cell entry (53). Furthermore, the pseudotype viruses with SSPE envelope glycoproteins could enter SLAM-negative cells including Vero and some neural cells, pointing to the existence of yet another entry receptor for SSPE viruses on certain cell types (53). Although it is poorly understood, cell tropism of MV is not determined solely at the cell entry level. Many internal cellular factors as well as viral mutations can affect the replication of MV (48, 49). Takeuchi and colleagues reported that mutations in the P or M gene of a Vero cell-isolate of MV (having genes for envelope glycoproteins identical to a B95a cell-isolate of MV) allow MV replication in Vero cells (55). Because the VSV pseudotype system cannot be used to study the replication of MV after cell entry, we characterized SSPE virus infection of different cell lines including those of neural origin by another approach.

Cells infected with SSPE viruses are usually defective in production of a cell-free progeny virus. Nevertheless, cell-free virus-like particles are needed to study their cell tropism for different cell lines. The freeze-thawing method (43) was unacceptable because our strains could not be reproducibly prepared and the titers were unsatisfactory. Preparation by treating infected cells with cytochalasin D (CD-VLPs) (2) enabled us to infect cell lines and thereby compare the infectivity differences between SSPE virus and MV.

Materials and Methods

Cells and viruses. Vero (African green monkey kidney) cells, Vero/SLAM (Vero cells expressing human SLAM) cells, and HeLa (human uterus cancer) cells were cultured at 35 C in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% fetal bovine serum (FBS) and 4% newborn calf serum. B95a (Epstein-Barr virus-transformed marmoset B lymphocytes) cells were cultured in RPMI 1640 medium supplemented with 5% FBS. Chinese hamster ovary (CHO) and CHO cells stably expressing human CD46 (CHO/CD46) (30) or human SLAM (CHO/SLAM) (57) were grown in RPMI 1640 medium supplemented with 10% FBS. Culture media for CHO/CD46 cells or CHO/SLAM cells were supplemented with 0.7 mg/ml of hygromycin B (Wako Pure Chemicals, Osaka, Japan) and 0.5 mg/ml of G418 (Calbiochem-Novabiochem, La Jolla, Calif., U.S.A.), respectively. In addition, 293T (human embryonic kidney), IMR-32 and SK-N-SH (human neuroblastoma), A172 (human glioblastoma), and U-251 (human astrocytoma) cells were cultured in DMEM supplemented with 10% FBS. For cultivation of IMR-32 and SK-N-SH cells, non-essential amino acid solution (Gibco BRL, Gaithersburg, Md., U.S.A.) was added to a final concentration of 1%.

A Vero cell-adapted laboratory MV strain (Nagahata, genotype C1) and a field MV strain (OCU98-1P, genotype D3) isolated from peripheral blood lymphocytes of a patient with acute measles by cocultivation with B95a cells, and SSPE virus strains isolated from the brains of patients with SSPE have been described previously (5, 36, 44). In this study, we used three SSPE viruses (Osaka-1 Fr/V, Osaka-2 Fr/V, and Osaka-3 Oc/V) isolated from the brains of three patients. These viruses are sibling viruses that were isolated with different cell lines (such as Vero or B95a cells) and from different regions of the brain (such as the frontal or occipital lobe). For isolation and designation of sibling viruses of each strain and detailed patient information, refer to (22, 44).

SSPE strains were usually maintained by repeated

(i.e., 20–30) subcultures of the infected cells without adding fresh cells after isolation. Biochemical and biological characteristics of SSPE viruses used in this study, such as nucleotide sequence of the viral genome, viral protein synthesis in infected cultures, extent of infectious cell-free virus production and neurovirulence in hamsters, were examined at this passage level (29, 42, 50, unpublished observations).

Preparation of infectious cell-free virus-like particles. To test cell tropism of SSPE strains of MV in different cell lines, it was necessary to obtain infectious cell-free virus particles. However, SSPE strains are highly cell-associated and rarely produce a cell-free virus. Cytochalasin D is known to affect the membrane of some cell types and to cause projections to form at the cell surface by a process called "zeiosis" (23). The knobs with a slender stalk are formed and contain cell components such as ribosomes normally resident in endoplasm. When cells infected with SSPE strains are treated with cytochalasin D, the resulting knobs are expected to contain viral nucleocapsids and display F and H glycoproteins on their surface, similar to virus budding at the cell membrane. It was expected that the knobs would be mechanically chopped off the surface by vigorous pipetting. In practice, SSPE virus was cultured in a 25-cm^2 flask until large syncytia were formed (usually two or three days after passage) and the culture medium was replaced by 2 ml of maintenance medium containing cytochalasin D (5 μg/ml; Sigma, St. Louis, Mo., U.S.A.). Cells were incubated at 35 C for 30 min and then pipetted vigorously. The culture medium was collected and clarified by centrifugation at 1,600×g for 15 min. The resulting supernatant fluid was stored at −85 C until use. We have successfully prepared CD-VLPs from cells infected with any of the three SSPE strains of MV. The titer of the CD-VLPs was around 10^3–10^4 plaque-forming units (PFU)/ml on confluent monolayer cultures of cells used for virus isolation. One of the CD-VLP stocks was filtrated through a 0.45-μm membrane filter and the titer was determined. The approximate size of CD-VLPs was estimated from the reduction in titer after filtration and was compared with that of the MV (Nagahata strain) particles produced naturally in the culture fluid.

Electron microscopy. Morphological examination of CD-VLPs was carried out by electron microscopy as previously described (31). A drop of the CD-VLP's stock was placed for 1 min on a 400-mesh collodion-coated copper grid covered with a carbon film. The resulting samples were negatively stained with 2% phosphotungstate (pH 6.4) for 1 min and were examined in a JEOL transmission electron microscope (JEM-1200EXII) at 80 kV.

Virus infection, syncytium formation, and antibody treatment. Monolayer cultures of various cell lines were prepared in 24-well tissue culture plates. One milliliter of culture media containing the MV strains or SSPE CD-VLPs (50 to 300 PFU/well) was added and then incubated at 35 C for 3 hr. The cells were washed with a culture medium to remove cytochalasin D in the stock solution of CD-VLPs, and the wash solution was then replaced by a fresh culture medium. Syncytial foci were counted at 48 hr post-infection for MV and at 72 hr post-infection for SSPE CD-VLPs using an inverted microscope.

For the antibody-blocking experiment, 300 μl/well of culture medium with or without mouse monoclonal antibodies was added to monolayer cell cultures in 24-well plates and pre-incubated for 30 min at room temperature. Antibodies (M177 and M160 [51] against human CD46 [10 μg] or IPO-3 [Kamiya Biomedical, Seattle, Wash., U.S.A.] against human SLAM [10 or 20 μg]) were used. Then, 700 μl of culture media containing SSPE CD-VLPs (50 to 300 PFU/well) was added without aspirating the media. Ten micrograms of M177 antibody but not M166 antibody was found to completely block syncytium formation by the Nagahata strain of MV.

Indirect immunofluorescence tests. Cells cultured on coverslips were infected, cultured for three or four days, rinsed once with phosphate-buffered saline (pH 7.4), and fixed with acetone. Cells were incubated with a monoclonal antibody against MV N protein at room temperature for 30 min and then stained with anti-mouse IgG rabbit antibody conjugated with FITC for 30 min at room temperature.

Flow cytometric analysis. Expression of CD46 and CD150 of various human cell lines was determined by flow cytometry (FACSCalibur, Becton Dickinson, Tokyo) after staining with a mouse monoclonal antibody against CD46 or CD150, followed by rabbit anti-mouse IgG conjugated with FITC.

Results

Syncytium Formation by SSPE CD-VLPs in Vero Cells and CHO Cells Expressing CD150

We previously characterized the cell entry receptors for SSPE by using VSV pseudotype virus expressing SSPE envelope glycoproteins (53). To further confirm the receptor usage by SSPE viruses under conditions similar to natural virus infection, we prepared virus-like particles by treating SSPE virus-infected cultures with cytochalasin D and mechanical pipetting. Electron microscopic examination of the CD-VLPs prepared from both infected and uninfected cells showed numerous spherical

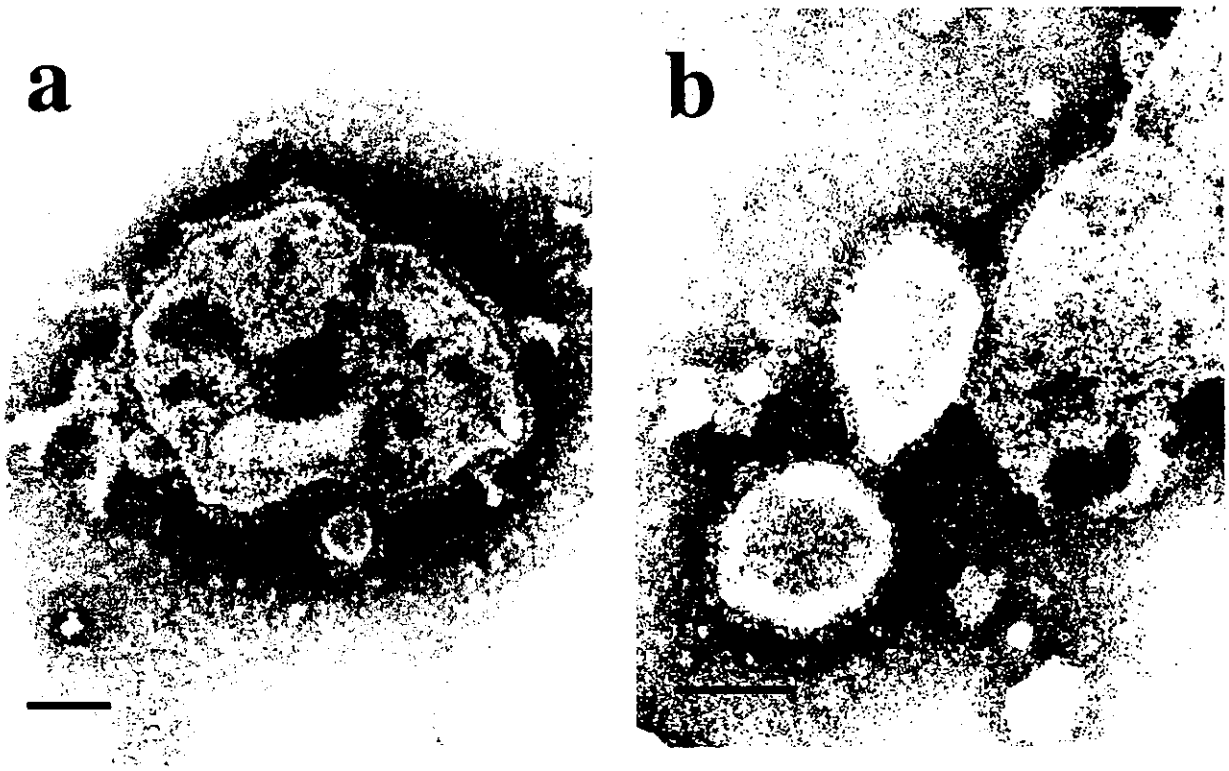

Fig. 1. Electron micrographs of CD-VLPs prepared from Vero cells infected with Osaka-1 Fr/V (a) or Osaka-2 Fr/V (b) strain. Bars represent 100 nm.

Table 1. Number of syncytium foci developed in CHO/CD46, CHO/SLAM, and Vero cells.

Virus	Strain	CHO/CD46	CHO/SLAM	Vero
Measles	Nagahata[a)]	13	135	109
SSPE	Osaka-1 Fr/V[b)]	0, 0	20, 25	460, 477
	Osaka-2 Fr/V[b)]	0, 0	99, 141	58, 81
	Osaka-3 Oc/V[b)]	0, 0	5, 4	141, 117

[a)] Syncytium foci was counted 48 hr post-infection.
[b)] Syncytium foci was counted 72 hr post-infection.

particles with diameters ranging from 100 to 1,000 nm. Some of the CD-VLPs from SSPE virus-infected cell cultures contained fine spikes similar to those of the authentic MV virion (Fig. 1). The titer of the CD-VLPs prepared from the Osaka-2 Fr/V strain dropped from 3.0×10^4 PFU/ml to 4.4×10^3 PFU/ml (approx. 85% reduction) after filtration with a 0.45-μm filter membrane. The titer of the Nagahata strain of MV decreased similarly after filtration. This data, together with electron microscopic observation, suggested that CD-VLPs were similar in size and surface structure to the MV virion.

We first examined the cytopathogenicity in Vero cells inoculated with SSPE CD-VLPs and in cells expressing known MV receptors, CD46 and SLAM. The Nagahata strain of MV, a laboratory Vero cell-adapted strain, was used for comparison and induced similar syncytia in size in Vero, CHO/CD46, CHO/SLAM cells (Table 1). The three SSPE CD-VLPs failed to induce CPEs in CHO/CD46 cells but were able to induce CPEs in both CHO/SLAM and Vero cells (Table 1). The number of syncytia produced in CHO/SLAM cells relative to the number in Vero cells varied among the infecting strains. The Nagahata strain induced a few more syncytia in CHO/SLAM cells than in Vero cells and very few foci in CHO/CD46 cells. The Osaka-2 CD-VLPs induced 2 to 3 times more syncytia in CHO/SLAM cells than in Vero cells. In contrast, the Osaka-1 CD-VLPs and the Osaka-3 CD-VLPs induced 20 to 25 times more syncytia in Vero cells than in CHO/SLAM cells. CHO/SLAM cells with syncytial CPE were positively stained with anti-MV N monoclonal antibody, whereas CHO/CD46 cells had no syncytial CPE and were negatively stained (data not shown). Infection of the SSPE CD-VLPs to CHO/SLAM cells was blocked by a monoclonal antibody against CD150. On the other hand, infection of the SSPE CD-VLPs to Vero cells was not blocked by a monoclonal antibody against CD46 (data not shown). These observations were consistent with the results of our previous studies using the VSV pseudotype system (53).

Table 2. Susceptibility of various cell lines to SSPE virus and measles virus

Cell lines	Expression[a] of		Osaka-1 Fr/V		Osaka-2 Fr/V		Osaka-3 Oc/V		Nagahata		OCU98-1P	
	CD46	CD150	Syncytium[b]	FA[c]	Syncytium	FA	Syncytium	FA	Syncytium	FA	Syncytium	FA
Vero	+	−	+	+	+	+	+	+	+	+	−	+[e]
B95a	+[d]	+	+	+	+	+	+	+	+	+	+	+
CHO	−	−	−	−	−	−	−	−	−	−	−	−
HeLa	+	−	+	+	+	+	+	+	+	+	−	−
293T	+	−	+	+	+	+	+	+	+	+	−	−
IMR-32	+	−	+	+	+	+	+	+	+	+	−	−
SK-N-SH	+	−	+	+	+	+	+	+	+	+	−	−
A172	+	−	+	+	−	+	−	+[e]	+	+	−	−
U-251	+	−	−	+	−	+	−	+	+	+	−	−

[a] Expression of CD46 and CD150 (+, positive; −, negative) was examined by flow cytometry.
[b] Syncytium formation (+, positive; −, negative) was observed under the inverted microscope.
[c] MV N protein expression (+, positive; −, negative) was detected by the immunofluorescent staining.
[d] B95a cell has a CD46 homologue that cannot be used as a receptor by most MVs because of the lack of SCR-1 domain (26, 39).
[e] Small syncytia were observed by the immunofluorescent staining.

Infection of Different Human Cell Lines with CD-VLPs Prepared from the Three SSPE Virus Strains

The system used here was applied to various human cell lines in order to determine other factors that may affect cell tropism. First, we rechecked the surface expression of CD46 and CD150 on these cell lines by flow cytometry (Table 2). Only B95a cells expressed CD150. In contrast, all cell lines except CHO cells expressed CD46. The CD-VLPs prepared from each of the three strains of SSPE virus-infected Vero cells were inoculated onto monolayer cultures of neural cell lines such as IMR-32, SK-N-SH, A172, and U-251, as well as onto monolayer cultures of Vero, B95a, CHO, HeLa, and 293T. As summarized in Table 2, all the SSPE CD-VLPs induced syncytial foci in most of the cell lines examined. Vero and B95a cells developed numerous large syncytia due to infection with the SSPE CD-VLPs (Fig. 2, a and b). The number of syncytial foci in HeLa, 293T, IMR-32, and SK-N-SH was less than 5% of that formed in Vero cells. In addition, the size of each syncytium in HeLa and 293T cells was much smaller than that of syncytia formed in Vero and B95a cells (Fig. 2, c and d). Syncytia formed in IMR-32 and SK-N-SH were similar in size to those formed in Vero cells (Fig. 2, e and f). Although infection of U-251 cells with the SSPE CD-VLPs was not obvious when judged by syncytium formation, clusters of infected single cells without cell fusion were demonstrated early in infection by immunofluorescent staining (Fig. 2, g). A172 cells showed different susceptibility to each of the SSPE CD-VLPs (Fig. 3). The CD-VLPs from the Osaka-1 Fr/V strain induced syncytial foci similar in size to those formed in Vero cells. The CD-VLPs from the Osaka-2 Fr/V strain did not induce syncytia but infection at the single cell level was demonstrated by immunofluorescent staining (Fig. 3, b and e). Though processes connected neighboring MV N protein-positive cells within clusters, cell margins could be clearly distinguished. The CD-VLPs from the Osaka-3 Oc/V induced microfusion that was observable by immunofluorescent staining (Fig. 3, c and f). As predicted by the receptor usage of the strain, the Vero cell-adapted Nagahata strain induced syncytia in all the cell lines except CHO (Table 2). In contrast, an MV field isolate, the OCU98-1P strain, infected the panel of cell lines to only a very limited extent. Vero cells (though infected with the OCU98-1P strain) lacked CPEs such as syncytium formation and cell rounding. Neither the human epithelial (HeLa and 293T) nor human neural (IMR-32, SK-N-SH, A172, and U-251) cell lines were infected with the OCU98-1P strain.

Discussion

Because most genes of SSPE viruses, especially the M and F genes, are highly mutated and cell-free virus production is impaired, SSPE virus must be rescued from infected brain cells by cocultivation with cells permissive for MV replication. Though epithelial cell lines (such as Vero cells) have been used for virus isolation, viruses have rarely been isolated. Identification of the biological properties of SSPE viruses (such as cell tropism) will be useful for understanding the molecular mechanisms of persistent infection of the CNS with MV. Cellular tropism of MV is broad, and factors determining it could lie at various stages of the MV life cycle. Little is known about the cell tropism of the SSPE virus. Cellular receptors are important determinants of viral tropism. We employed the VSV pseudotype system to identify the

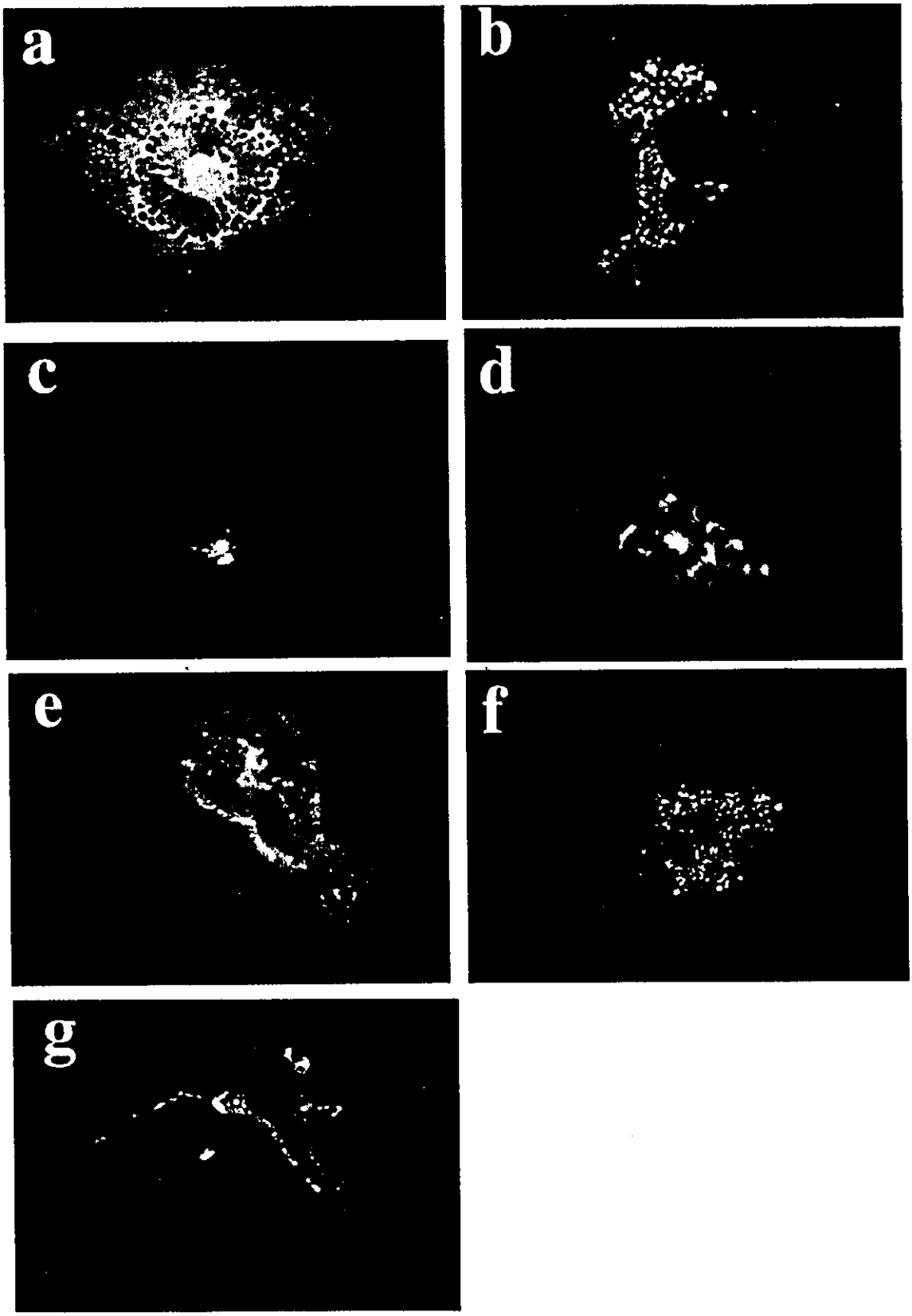

Fig. 2. Cells infected with CD-VLPs prepared from Osaka-2 Fr/V virus-infected Vero cells were evaluated by immunofluorescent staining with monoclonal anti-N antibody. Vero (a), B95a (b), HeLa (c), 293T (d), IMR-32 (e), SK-N-SH (f), and U-251 (g), cells were fixed and stained three or four days after infection. Magnifications are ×65 for (a) to (f), ×130 for (g).

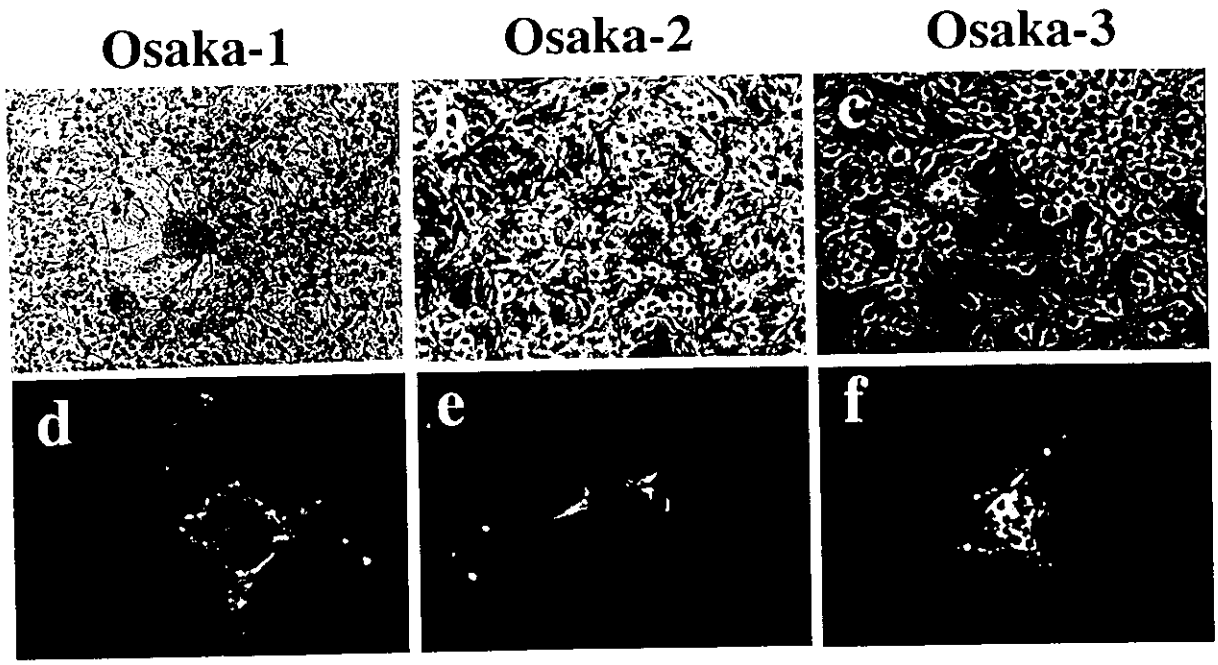

Fig. 3. A172 cells infected with CD-VLPs prepared from Vero cells infected with Osaka-1 Fr/V (a, d), Osaka-2 Fr/V (b, e), and Osaka-3 Oc/V (c, f) strains. N protein expression was examined by indirect immunofluorescent staining (d–f).

receptors used by SSPE viruses for cell entry and our results suggested entry via some previously unidentified molecule(s) on Vero, B95a, human B-lymphocytes, and some human cell lines of neural origin (53). To obtain more information about the cell tropism of SSPE virus, artificial virus-like particles obtained from cells infected with three strains of SSPE virus were tested for their ability to infect various human cell lines including cell lines of neural origin. We found that CD-VLPs from SSPE virus-infected cells were similar morphologically and could be used like ordinary MV particles to infect cells.

We first confirmed our previous observation that SSPE virus could use SLAM but not CD46 as a cell entry receptor (53). Antibody blocking experiments indicated the existence of previously unidentified receptors on Vero cells, as did our experiments that used VSV pseudotype virus displaying SSPE envelope glycoproteins (53). Receptor molecule(s) other than CD46 and CD150 for MV entry have recently been suggested by other investigators (1, 24, 56). It is unknown whether the molecule used for entry into Vero and Jarkat cells (24), human vascular endothelial cells (1), and human primary small airway epithelial cells (56) is unique to each cell line and can be used by different MV strains. If the unidentified molecule(s) for MV entry are closely related to each other, the molecule(s) must be ubiquitously distributed among various cell types and different host species. Notably, the three SSPE strains used in this study have strong neurovirulence in 3-week-old hamsters, and this can be demonstrated by intracerebral inoculation of just a small amount of their CD-VLPs (29, unpublished observations). In contrast, the results of Niewiesk et al. (41) in transgenic rats show that CD46 expression cannot overcome the intracellular block of MV Edmonston strain replication, supporting the requirement of nonentry-supporting internal cellular factors for MV replication. One such factor might be IFN, and in fact, α/β IFN receptor-knockout mice have been reported to be susceptible to Edmonston MV infection (38).

The ability to induce syncytia in glial cells such as A172 cells was different among SSPE virus strains. Syncytial formation was induced by the Osaka-1 CD-VLPs, and less efficiently by the Osaka-3 CD-VLPs, but not by the Osaka-2 CD-VLPs. It should be noted that the Osaka-1 and the Osaka-3 CD-VLPs induced more syncytia in Vero cells, indicating that the Osaka-1 and the Osaka-3 strains can use more efficiently an as yet unidentified receptor for syncytium formation. Binding affinity between the SSPE H glycoprotein and the receptor molecule may have some relation to the ability to induce syncytium in A172 cells. Alternatively, other viral proteins (F, M or P protein) may have been involved in this failure to induce syncytium by the Osaka-2 CD-VLPs. The infection of A172 as well as U-251 cells with the SSPE CD-VLPs at the single cell level and the failure to form syncytia is unusual for MV infection *in vitro*. We have observed a similar phenomenon (failure to form

large syncytia) in Vero cells infected with the Masusako strain, and Kouomou and Wild reported a similar finding (34). MV infection in human astrocytoma cells was described previously by Duprex et al. (18, 19). By means of an Edmonston-based recombinant virus construct that encoded green fluorescent protein, they showed that cell-to-cell spread took place through the cells' processes during early infection of GCCM cells (18) and that syncytia formed extensively in GCCM and U-251 cells (19). Although they did not consider the role of the cell receptor in the infection process, CD46 is likely to be involved in this process. Our CD46-using Nagahata strain could also infect and induce syncytia in A172 and U-251 cells. Therefore, it is possible that the failure of the SSPE CD-VLPs to induce syncytia in these cell lines is not due to a defect in the internal cellular factors of these lines, but rather, to the inability of the cellular receptor to interact with the envelope proteins of the SSPE virus. Alternatively, reduced replication efficiency might have resulted in the failure to produce viral proteins needed to induce cell fusion. Persistent infection of these A172 cells by the Osaka-2 Fr/V strain was demonstrated after 10 days by the appearance of syncytial foci in cocultures of the infected A172 and Vero/SLAM cells (unpublished data). The titer, however, decreased gradually with time, indicating the unsuitability of A172 cells for efficient growth of SSPE virus. We did not detect IFNs in the culture media of the infected A172 and U-251 cells, and anti-IFN antibodies did not affect infection (unpublished data). Therefore, other internal host factors might have been involved in the inefficient viral replication. The failure to activate NF-κB, type I IFN, and MHC class I in neuroblastoma cells (14, 15, 21) may account for the more efficient growth of SSPE virus in neuroblastoma than in glial cells. Further studies are needed to understand the viral and/or host cellular factors that are responsible for the strain differences in SSPE viral replication in neural cells. When the amino acid sequences of the two MV strains, IC-B and IC-V, isolated with B95a and Vero cells, respectively, were compared, H and F proteins were identical, but there were a few amino acid differences in the P, M, and accessory V and C proteins (54, 55). We have compared the amino acid sequence of viral proteins and the neurovirulence in hamsters of two Osaka-2 sibling viruses (5, 22, 29). The Vero-isolate Fr/V of the Osaka-2 strain used in this study and the B95a-isolate Fr/B of the same strain both encode an H protein of the same amino acid sequence. The F protein of the Fr/B sibling virus had only two additional changes in its amino acid sequence compared with that of the Fr/V sibling virus. Our preliminary results showed that the Osaka-2 Fr/B sibling virus did not infect IMR-32, A172, and U-251 cells. This suggests that factors other than the entry receptor are active during Osaka-2 SSPE virus replication in neural cells. MV recombinants containing single or a combination of mutations identified from the sequence analysis of SSPE strains will provide clear evidence that virus infection of neural cells involves viral factors. Some mutations found in SSPE viruses may contribute to the neurovirulence and persistence of infection in the brain. This is supported by the fact that a recombinant MV containing the matrix gene of the Biken strain of SSPE virus replicated slowly in the brain of transgenic mice (45). Brains infected with recombinant MV containing F proteins having cytoplasmic tails (similar to the tails of F proteins of SSPE viruses) also manifested modified virus replication in the same transgenic mouse model (8). These alterations might promote virus spread and survival in the brain. Further studies to identify internal host factor(s) responsible for SSPE viral cell tropism and to identify novel molecule(s) associated with SSPE virus entry into cells are needed, thereby providing new insights into the molecular mechanism of SSPE pathogenesis.

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原　著

未熟児、新生児のけいれん重積治療における リドカインの使用経験

Treatment with Lidocaine for Status Epilepticus in Low-Birth-Weight Infants and Neonates

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小林憲昭　宮本雄策　福田美穂

要旨：未熟児、新生児のけいれん重積に対するLidocaine(Lid)投与の効果を後方視的に検討した。Lidは生後0～3日の間に使用した。対象者は「けいれんが持続または群発した未熟児、新生児で通常の抗けいれん薬投与を含めけいれんに対して何らかの対処を必要とした者のうち、けいれんが停止せずLidを使用した者」とした。最初にLid(静注用キシロカイン)2 mg/kg/doseを静注後、2～4 mg/kg/hrで持続静注した。対象者の生下時体重は580～3,200 g、在胎週数は25～40週の間に分布していた。9例中5例(56%)に有効であったが、うち1例は徐脈のため投与中止となった。有効例は、低酸素性虚血性脳症の例が多く、染色体異常や脳形成異常に伴うものには無効であった。副作用は併用薬との薬物相互作用が疑われた。未熟児、新生児のけいれん重積に対しても今後症例によりLidの使用を検討すべきと考えられた。

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Key Words：lidocaine, status epilepticus, low-birth-weight infant, neonate
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はじめに

Lidocaine (lid)は1948年に開発されて以来、局所麻酔薬、抗不整脈薬として広く使用されている。また、その臨床的抗けいれん作用は、1955年にBernhardらによって初めて報告された[1]。それ以降、けいれん重積症、難治てんかんなどにも使用されその有効性が報告されている[2]。また最近は新生児けいれんにも使用されている。しかし、未熟児、新生児のけいれん重積に対する十分なデータは集積されていない。そこで、今回われわれは未熟児、新生児のけいれん重積に対してLidを使用し後方視的にその効果、副作用について検討したので報告する。

対象および方法

対象は最近5年間に当院新生児集中治療室に入院し、低酸素性虚血性脳症、脳室内出血、滑脳症、

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Table 1 Effective Cases

Patients	Diagnosis	Maintenance dose (mg/kg/hr)	Duration of Lid therapy (days)	Blood concent-rations* (μg/ml)	Seizure type	Adverse effects	Prognosis
M.O.	HIE Intraventricular hemorrhage	3	4	3.3	GT	None	Symptomatic partial epilepsy Developmental delay
Y.S.	HIE	3	3	3.5	GT	None	Good
F.K.	HIE Extremely low birth weight	3	3	—	GT	None	Died
K.N.	Bacterial meningitis (group B streptococcal)	2	2	5.5	GT	None	Developmental delay
R.S.	HIE Very low birth weight	4	0.125	—	FC	Bradycardia	Symptomatic partial epilepsy

HIE：hypoxic ischemic encephalopathy, GT：generalized tonic seizures, FC：focal clonic seizures, *24hrs after Lid administration

染色体異常などに伴いけいれん重積を呈した未熟児、新生児9例とした。今回のけいれん重積症例は、生後0～3日の間に15分以上にわたり連続または群発するけいれんをみとめ通常の抗けいれん薬であるフェノバルビタール（PB）、ジアゼパム（DZP）、フェニトイン（PHT）の投与を含め、けいれんに対して何らかの対処（人工呼吸器管理やグルコース、カルシウム投与など）を必要とした者のうちけいれんが停止せずにLidを使用した者とした。Lidの使用方法は、最初に静注用キシロカイン2 mg/kg/doseを静注後、2～4 mg/kg/hrで持続静注した。対象の出生時体重は580～3,200 g（平均1,920 g）、在胎週数は25～40週（平均32週）の間に分布していた。またApgar scoreの平均は5.2であった。基礎疾患は、低酸素性虚血性脳症、脳室内出血、滑脳症、染色体異常などであった。発作型はVolpeの分類[3]に従って判定した。

結　果

初回Lid投与後にけいれんが消失し、持続静注後も24時間以上再発しなかった例を有効とした。9例中5例が有効とみとめられた。そのうち4例はLidを3～7日間持続した後減量し他の維持療法に変更できた。1例は投与開始後3時間で徐脈となり投与中止となった。中止後は再度けいれん重積となりペントバルビタール（PTB）を使用した。持続できた4例中1例は、けいれんは停止していたが日齢14に呼吸障害、循環障害が改善せず死亡した。これら5症例のまとめをTable 1に示す。Lidの維持投与量の平均は3.1 mg/kg/hr、平均投与日数は4.5日であった。投与後24時間のLid血中濃度は3.3～5.5 μg/mlであった。他の明らかな副作用はみとめなかった。転帰として、てんかんを残したものが2例みとめられた。無効例はLid投与後もけいれん発作が不変だったものとした。9例中4例が無効であった。けいれん発作が悪化した例はなかった。これら無効例の内わけをTable 2に示した。無効例の基礎疾患は、脳室内出血、染色体異常、滑脳症などであった。無効例ではその後PTBを投与したものが3例、他の抗けいれん薬（ゾニサミド）を投与したものが1例あった。

Table 2 Non Effective Cases

Patients	Diagnosis	Seizure Type	Adverse Effects	Finally Effective Drugs	Prognosis
Y.N.	Intraventricular hemorrhage	GT	None	Pentobarbital	DM
Y.W.	Intraventricular hemorrhage Very low birth weight	FC	None	Zonisamide	Symptomatic partial epilepsy, DM
S.R.	Lissencephaly Chromosome adnormality	GT	None	Pentobarbital	Symptomatic generalized epilepsy, DM
E.S.	Lissencephaly	GT	None	Pentobarbital	Symptomatic partial epilepsy, DM

HIE：hypoxic ischemic encephalopathy, GT：generalized tonic seizures, FC：focal clonic seizures, DM：developmental delay

考　察

Lidの小児への投与は1980年代より報告され、80年代後半には新生児にも使用され有効な結果を得ている[4]。しかしLidのけいれん重積治療に関しては十分なデータが集積されていないのが現状である。

Lidは全般発作には無効で、局所性焦点を有する部分てんかんに有効であったことから大脳皮質における神経興奮抑制作用が、その抗けいれん作用発現理由の一つとして考えられている[5]。またその生理機序としてNaチャネルに作用して膜の興奮性を低下させるという説[6]、神経細胞のミトコンドリアの代謝を低下させ、神経細胞興奮を抑え、大脳皮質の酸素消費量を減少させるという説[7]などがある。また近年、欠神発作やミオクロニー発作などの全般発作にも有効との報告もある[8]。しかし複雑部分発作を有する場合は、けいれんの誘発に注意すべきであると言われている。前頭葉てんかんやLennox-Gastaut症候群でのけいれんの悪化も経験されている。また大量投与では抑制性神経細胞が抑制され、扁桃体から広がるけいれん誘発作用をもつ[9]。

今回の未熟児、新生児のけいれん重積では、有効例はほとんどが低酸素性虚血性脳症に伴うもので発作型は全般発作が主体であり、従来の報告でのLidの発作抑制機序はそのまま当てはまらないと考えられた。Lidの使用方法として初期のころは、1～2 mg/kg/doseのone shot静注の方法がとられていたが、1980年代以降は2 mg/kg/dose iv後に2～4 mg/kg/hrで持続静注する方法が一般的である。今回もこの使用法で行い大きな副作用はみられなかった。持続静注量については血中濃度と臨床効果をみながら漸減、漸増することが多い。投与量と血中濃度は、ほぼ相関するという報告が多い[10]。有効血中濃度は1～6 μg/ml の範囲で、副作用の出現は必ずしも血中濃度と相関するわけではないが、副作用の一部は5 μg/ml を越えるものが多いといわれている。

Lidを抗けいれん薬として使用した時の副作用の出現は、過去の文献をまとめた報告によると、258症例中16例にみられている[11]。その内訳は、神経系では、けいれん誘発が4例(新生児1、乳児2、幼児1)、ふらつきが3例、軽度眠気が2例、幻視幻聴が1例、筋トーヌス低下が2例であった。循環器系では、徐脈が3例（新生児1、乳児2)、心停止が1例であった。今回の使用例でも徐脈が1例みられた。この例はLidとグルコン酸カルシウムを併用していたが、徐脈はLid中止、グルコン酸カルシウム減量後速やかに改善したため原因は薬物相互作用が疑われた。Lidでは心抑制などの副作用は比較的起こりにくいといわれているが、

急速に静注した時や、肝血流を低下させるような薬剤または循環器系薬剤と併用した時に、血圧低下、不整脈等の副作用が出現しやすいのではないかと考えられる。

Lid使用にあたっては、大量投与による血中濃度の上昇、麻酔薬や循環器系薬剤との相互作用、宿主側の心機能や肝機能に十分注意する必要があるものと思われる。Lidの副作用の報告は、過去の文献上では未熟児、新生児に限ったものはなく様々な年齢層を含んだものとなっている。前述したように過去の258症例のまとめでは有効率は約80%、副作用は約6%と報告されている[11]。今回の副作用は9例中1例（約11%）とやや高率であった。他の薬剤との比較では最近ミダゾラムもよく新生児けいれんに使用されているがやはり未熟児、新生児に限った報告はない。平成14年度の後方視的多施設共同研究のまとめでは、ミダゾラムの有効率63.5%、副作用13.2%と報告されている[11]。Lidとミダゾラムの副作用には大きな差はないと考えられる。未熟児、新生児のけいれん重積治療におけるLidの位置づけは現状では、通常の抗けいれん薬（DZP、PB、DPHなど）が無効であった場合のミダゾラムを含めた選択肢の一つと考えて良いと思われる。今後は全国調査などを踏まえた小児のけいれん重積治療ガイドラインが作成されることが望ましい。今回の検討により、Lidは未熟児、新生児の低酸素虚血性脳症に伴うけいれん重積に有効で、とくにけいれん群発例では持続静注によりけいれんを継続して抑制できることがわかった。さらに、静注してから効果発現は速やか（通常、血中濃度は注入直後に最高濃度を示す[11]）で効果判定が行いやすく、臨床症状の改善だけでなく、全例施行したわけではないが一部の例では脳波の改善もみとめられた。また、もうひとつの利点として、半減期が短いため副作用出現時も投与の中止により速やかに副作用が改善できると考えられた。未熟児、新生児のけいれん重積に対しても、症例を選んで使用すればLidは有用な薬剤と思われた。

結　論

未熟児、新生児のけいれん重積に対するLid投与の効果を後方視的に検討した。9例中5例（56%）に有効であったが、うち1例は副作用（徐脈）のため投与中止となった。有効例は、低酸素性虚血性脳症の例が多く、染色体異常や脳形成異常に伴うものには無効であった。副作用は併用薬との薬物相互作用が疑われた。

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症例報告

てんかんを合併した原発性小頭症の3例

Three Cases of Primary Microcephaly Associated with Epilepsy

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村上浩史　宮本雄策　福田美穂

要旨：てんかんを合併した原発（真）性小頭症の3例を経験した。染色体異常症、奇形症候群や先天性感染症、周産期の異常などに伴う二次性の小頭症に合併するてんかんの報告は数多くみられるが原発性小頭症に合併したてんかんのまとまった報告はない。3症例とも生下時から正常頭囲より－3 S.D. 以下の小頭をみとめた。頭蓋の特徴としては、前頭、後頭部の低形成、前額部の sloping、尖状頭がみられ、他の脳奇形、染色体異常症、先天性感染症などが否定されたため原発性小頭症と診断した。それぞれ、生後9カ月、10カ月、1歳9カ月にてんかんを発症した。すべて症候性局在関連性てんかんであり、1例は薬物抵抗性の難治てんかんであった。発作型は複雑部分発作と二次性全般化発作を示した。全例とも精神遅滞を伴っていたが、その程度はさまざまであった。　**てんかん研究　2004；22：201-205**

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はじめに

頭の大きさは便宜上頭囲の大きさにより判定される。頭囲の増大には脳の成長と頭蓋縫合の開存が必要であり、このどちらかに障害があると頭囲の増大は停止もしくは遅延する。増大が停止すると小頭症となっていく。小頭症は正常頭囲より－3.0 S.D.以下のものを総称する疾患名である。小頭症にはてんかんの合併が多いといわれるが、過去の報告例はほとんどが先天性脳奇形、染色体異常症、先天性感染症などに伴うものであり、原発性小頭症に伴うてんかんのまとまった報告はなく、合併はまれと思われる。今回、われわれは、てんかんを合併した原発性小頭症を3例経験したので文献的考察を加えて報告する。

症　例

症例1：11カ月の男児
主訴：小頭、けいれん
家族歴：特記すべき事なし
妊娠・分娩歴：妊娠中より胎児エコーにて小頭を指摘されていた。在胎38週、経腟分娩にて出生し仮死は認めなかった。出生体重2,154 g、身長50 cm、頭囲27 cm（－4.0 SD）であった。

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