**Table 2. Causative factors in 34 patients with Rasmussen's syndrome at the Shizuoka Institute of Epilepsy and Neurological Disorders.**

| | EPC type | Non-EPC type | Total |
|---|---|---|---|
| Age of onset (years) | 6.3 ± 5.6 | 8.7 ± 8.0 | 7.4 ± 6.7 |
| Preceding infections | 8 (40.0%) | 5 (35.7%) | 13 (38.2%) |
| • Fever only | 4 | 1 | 5 |
| • Upper respiratory infection | 2 | 1 | 3 |
| • Influenza | 1 | 2 | 3 |
| • Mycoplasma | 0 | 1 | 1 |
| • Aseptic meningitis | 1 | 0 | 1 |
| Vaccination | 1 (5.0%) | 1 (7.1%) | 2 (5.9%) |
| Head trauma | 2 (10.0%) | 1 (7.1%) | 3 (8.8%) |
| None | 9 (45.0%) | 7(50.0%) | 16(47.1%) |
| Total | 20 | 14 | 34 |

*EPC: Epilepsia partialis continua.*

patients [11]. Peripheral blood lymphocytes in patients are sensitized to GluRε2 [13]. Heterogeneous autoantibodies against neuronal molecules (including GluR3, GluRε2, neuronal acetylcholine receptor α-7, and munc-18) and glial cells are detected in Rasmussen's syndrome [12,27]. Autoantibodies against GluRε2 have epitopes predominantly in intracellular domains and show epitope spreading evolutionally [12]. These data suggest that humoral autoimmunity mediated by autoantibodies is not the primary factor causing Rasmussen's syndrome, and that cellular autoimmunity mediated by CTLs plays a primary role in the development of this syndrome (Figure 2). For treatment, the author considers the choice of tacrolimus to suppress activation of T cells in patients with dominant hemisphere involvements [28], although functional hemispherectomy is the first choice in patients with nondominant hemisphere involvements. Autoantibodies against neural molecules seem to be produced after the onset of Rasmussen's syndrome, but those autoantibodies may affect the pathological processes after onset of Rasmussen's syndrome by the function proved in the studies of autoantibodies against GluR3 [10,29–33].

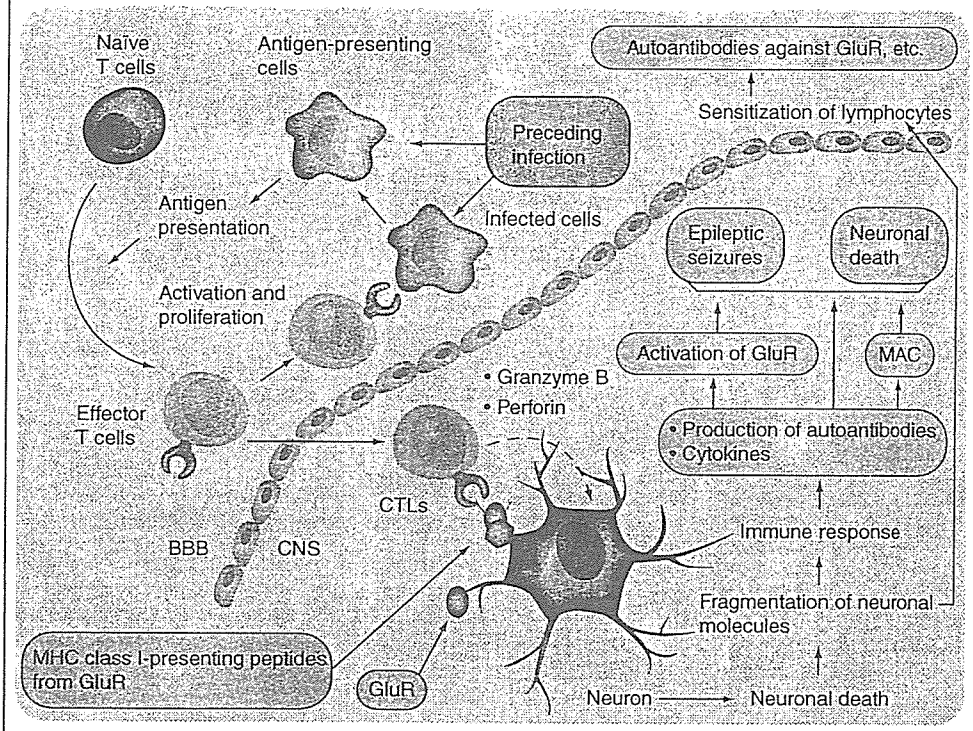
## Rasmussen's syndrome & molecular mimicry determined by human leukocyte antigen

After the T-cell receptors (TCRs) on CTLs recognize both the human leukocyte antigen (HLA) class I molecule and its binding peptide expressed on antigen-presenting cells (APCs), CTLs are activated into effector CTLs that can invade the CNS easily. If, through molecular mimicry and TCR redundancy, the CTLs activated by microbial peptides are able to recognize the HLA class I molecule and its binding peptide from neuronal molecules on neurons, then the CTLs activated by infection may induce apoptosis of the neurons. Therefore, HLA class I is one of the key molecules determining the autoimmune mechanisms underlying the process from infection to the development of Rasmussen's syndrome.

The author studied the genotypes of HLA class I in 16 Japanese patients with Rasmussen's syndrome (9 with EPC type and 7 with non-EPC type) by polymerase chain reaction amplifications. HLA-A*2402 was a common genotype among Japanese people (36.5% of the Japanese population) and was found in 77.8% of EPC type patients ($p = 0.016$). HLA-A*0201 was found more frequently in non-EPC type patients (42.9%) compared with the Japanese population (10.7%) ($p = 0.033$). HLA-A*2601 was also found more frequently in non-EPC type patients (42.9%) compared with the Japanese population (11.3%) ($p = 0.038$). HLA-B*5201 was found more frequently in EPC type patients (33.3%) compared with the Japanese population (10.9%) ($p = 0.070$). HLA-B*4601 was found more frequently in non-EPC type patients (28.6%) compared with the Japanese population (3.4%) ($p = 0.025$).

HLA-B*4601 binds peptides with the following motif: x-[M]-x (5–7)-[Y/F] (x: free amino acid; M: methionine; Y: tyrosine; F: phenylalanine). Database analyses revealed the presence of this motif in various viral molecules and neural

**Figure 2. Primary roles of cytotoxic T cells in the mechanism of developing Rasmussen's syndrome.**

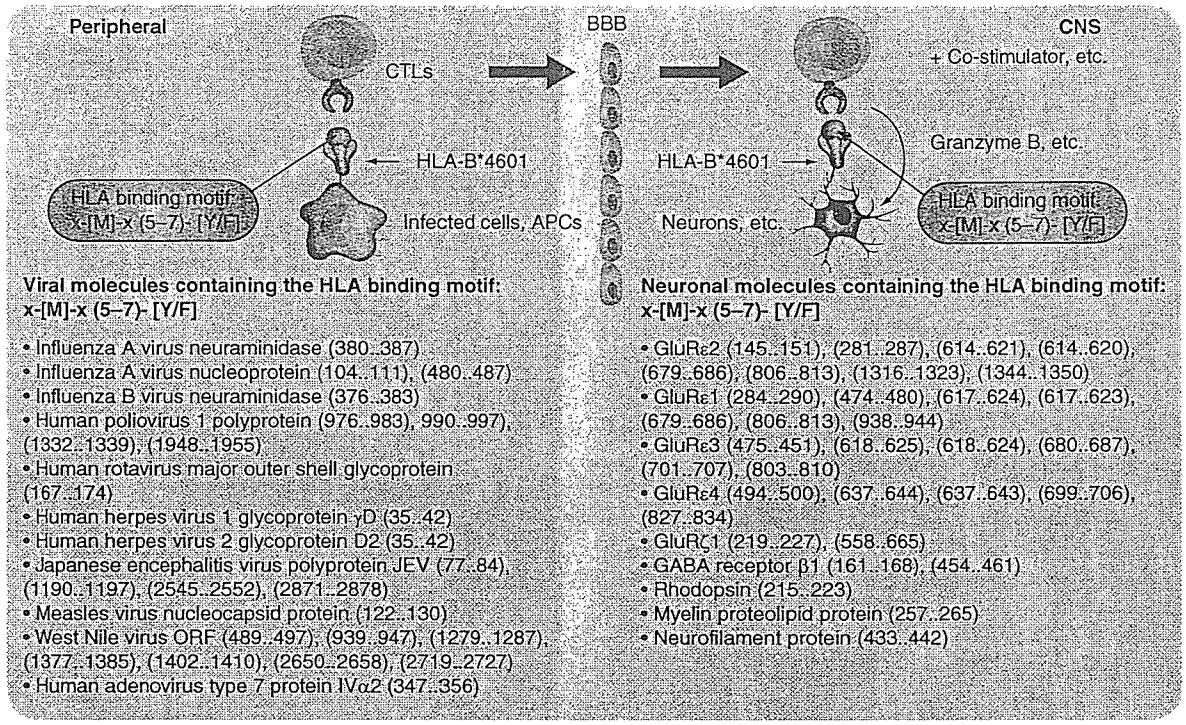



Effector CTLs, activated by preceding infections or vaccinations, cross the BBB to reach the CNS and cross-react with neurons, resulting in apoptosis. Neuronal death leads to the production of autoantibodies against CNS molecules and cytokines, which may contribute to further neuronal death or epileptogenesis. Fragmented neuronal molecules reach the systemic circulation and sensitize lymphocytes, resulting in the production of autoantibodies in the blood.
BBB: Blood–brain barrier; CTL: Cytotoxic T lymphocyte; GluR: Glutamate receptor; MAC: Membrane attack complex; MHC: Major histocompatibility complex.

molecules (Figure 3). If patients with HLA-B*4601 are infected by influenza A virus, the peptide from the neuraminidase of influenza A (AMTDWSGY) may bind to HLA-B*4601. TCRs of CTLs recognize HLA-B*4601 and the neuraminidase peptide (influenza A) on APCs, and are activated. Theoretically, the activated CTLs invade the CNS and may react with neurons possessing HLA-B*4601 and its binding peptide containing the (x-[M]-x (5–7)-[Y/F]) motif, due to degeneracy in TCR recognition, and may cause apoptosis of the neurons [34]. Peptides having the motif are found in various neuronal molecules including GluRε2 (IMEEYDWY, DMLSEHSF, IMVSVWAF etc.) and myelin proteolipid protein (FMIA ATYNF). Flexible interactions between TCRs of CTLs and various ligands on HLA-representing CNS molecules may facilitate broad CNS involvement of Rasmussen's syndrome. Alternatively, flexible interactions between CTLs and microbial molecules may enable aggravation of symptoms by infections or vaccinations. T-cell clones from Type 1 diabetes patients react with microbial mimicry peptides [34]. The cross-reactivity of CTLs from many Rasmussen's syndrome patients with microbial and neural molecules will be investigated in the future [24].

In patients with HLA-B*4601, neurons expressing *N*-methyl-D-aspartate (NMDA)-GluRs may be mainly involved in the interaction with CTLs as NMDA-GluRs possess the x-[M]-x (5–7)-[Y/F] motif in many domains (Figures 3 & 4). In patients with HLA-A*0201, the peptide from hemagglutinin of influenza A binds with the HLA-A*0201 molecule. CTLs activated by this complex may cross-react with neurons expressing NMDA-GluRs, γ-aminobutyric acid

**Figure 3. Database analyses of the motif that binds to HLA-B*4601 in microbial and neural molecules.**




Viral infections lead to the expression of certain peptides from viral molecules with HLA class I molecule. T-cell receptors of CTLs recognize the peptide and HLA class I molecules, resulting in the generation of effector CTLs. In individuals with HLA-B*4601, the binding motif is x-[M]-x (5–7)-[Y/F] (x: free amino acids; M: methionine; Y: tyrosine; F: phenylalanine). Database analyses revealed many viral molecules containing the motif, as shown on the left [102]. The numbers in parentheses are sequence numbers indicating the sites of the motif. Theoretically, the effector T cells that cross the BBB to reach the CNS may cross-react with neurons expressing the same motif on the HLA class I molecule in the presence of specific conditions, such as co-stimulators. Database analyses revealed many neural molecules containing the motif, as shown on the right, and many *N*-methyl-D-aspartate-GluRs contain the motif in several domains.
APC: Antigen-presenting cell; BBB: Blood–brain barrier; CTL: Cytotoxic T lymphocyte; GABA: γ-aminobutyric acid; GluR: Glutamate receptor; HLA: Human leukocyte antigen; ORF: Open reading frame.

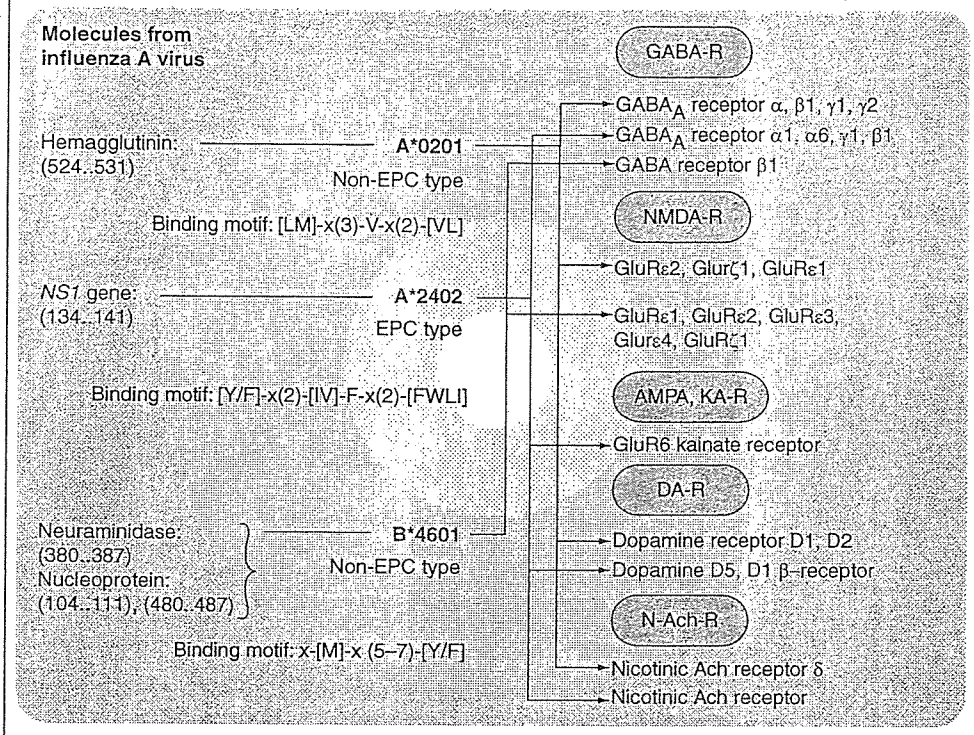
(GABA) receptors, dopamine receptors and other receptors, as these receptors have the motif that can bind with HLA-A*0201 (Figure 4). In patients with HLA-A*2402, the peptide from NS1 of influenza A binds with HLA-A*2402 and the resulting activated CTLs may cross-react with neurons expressing GABA receptors, dopamine receptors and adenosine monophosphate acid (AMPA) receptors, since these receptors, but not NMDA-GluRs, have the motif that can bind to HLA-A*2402. The HLA may explain the variable clinical symptoms manifested in patients infected by the same agent.

## Acute viral encephalitis & autoimmunity

Acute viral encephalitis is an important disease causing infection-associated epilepsies. Pathological mechanisms of acute viral encephalitis are divided into the primary viral infection mechanism and the parainfectious secondary autoimmune mechanism. In order to improve prognosis of acute encephalitis, elucidation of autoimmune mechanisms in the secondary autoimmune encephalitis is desirable.

The author analyzed autoantibodies against GluRε2 in the cerebrospinal fluid (CSF) of 46 patients with acute encephalitis or encephalopathies, categorized into localized encephalitis (24 patients) and widespread encephalitis (22 patients) by clinical symptoms in the initial stage [12,35]. Patients with localized encephalitis showed psychic symptoms (illusions, anxiety and distraction etc.), solitary seizures and/or very mild impairment of consciousness in the initial stage, and gradually evolved to severe conditions with convulsive status and a loss of consciousness. The mean age of onset was 33.0 years ± 18.4. A total of 21 out of 24 patients had a better

**Figure 4. Hypothetical cross-reactions between cytotoxic T cells expressing various molecules from influenza A virus and neurons expressing various neuronal molecules in individuals with different HLA class I genotypes.**




Database analyses showed that the motif that binds to each HLA class I type is found both in the influenza A molecules and neuronal molecules. Each HLA class I type had characteristic distributions of neuronal molecules containing the motif.
AMPA: Adenosine monophosphate acid; DA-R: Dopamine receptor; EPC: Epilepsia partialis continua; GABA-R: γ-aminobutyric acid receptor; HLA: Human leukoctye antigen; KA-R: Kainate receptor; N-Ach-R: Nicotinic-Ach-receptor; NMDA-R: *N*-methyl-D-aspartate receptor.

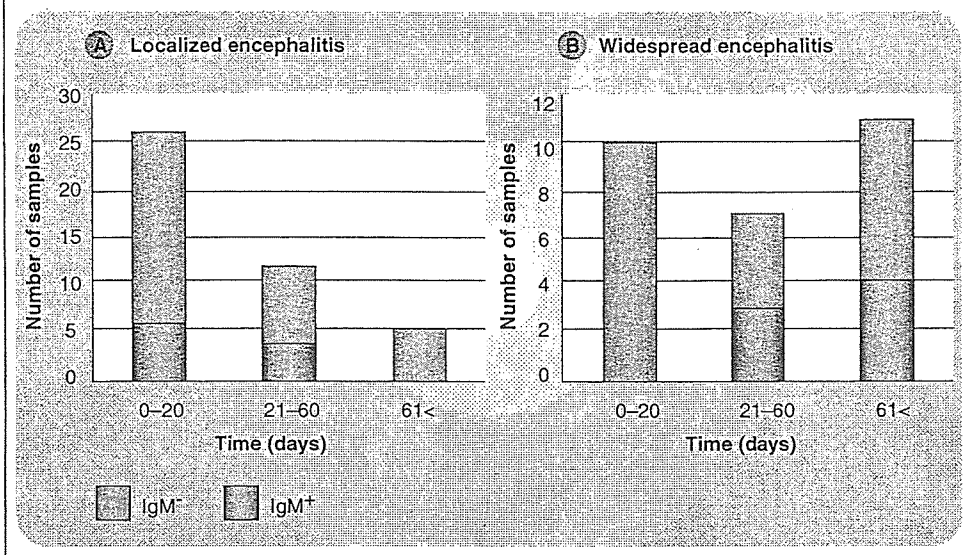
outcome, but three patients had sequelae. However, patients with widespread encephalitis showed a profound loss of consciousness and or convulsive status in the initial stage. The mean age of onset was 10.2 years ± 15.3. Six out of 22 patients were cured with better outcome, but 16 patients had sequelae.

In patients with localized encephalitis, immunoglobulin (Ig)M autoantibodies against GluRε2 tended to appear in CSF in the acute stage (0–20 days after onset of neurological symptoms) or recovery stage (21–60 days after onset of neurological symptoms) of encephalitis (Figure 5). In patients with widespread encephalitis, IgM autoantibodies against GluRε2 in CSF tended to appear in the recovery stage (21–60 days after onset of neurological symptoms) or chronic stage (>60 days after onset of neurological symptoms) of encephalitis. These data may suggest that GluR autoimmunity contributes to the onset of localized encephalitis and development of sequelae in widespread encephalitis. In patients with widespread encephalitis, the presence of autoantibodies against GluRε2 in CSF correlates significantly with onset of epilepsy after encephalitis ($p = 0.01$, Fisher's exact probability test) and with mental impairment ($p = 0.03$, Mann–Whitney U test). Therefore, autoantibodies against GluRε2 may have a causal relationship with epileptogenesis after widespread encephalitis.

## Hypothetical common autoimmune processes leading from neuronal damage to neurological sequelae

In neurological diseases, irrespective of the etiological factors (infections and vaccinations etc.), common symptoms and sequelae (such as

**Figure 5. IgM autoantibodies against GluRε2 in patients with acute viral encephalitis.**




**(A)** Shows frequencies of IgM autoantibodies against GluRε2 in cerebrospinal fluid from patients with localized encephalitis and **(B)** shows the frequencies in patients with widespread encephalitis. Samples are categorized into acute (0–20 days after neurological symptom onset), recovery (21–60 days after neurological symptom onset) and chronic (>61 days of neurological symptom onset) stages.
GluR: Glutamate receptor; Ig: Immunoglobulin.

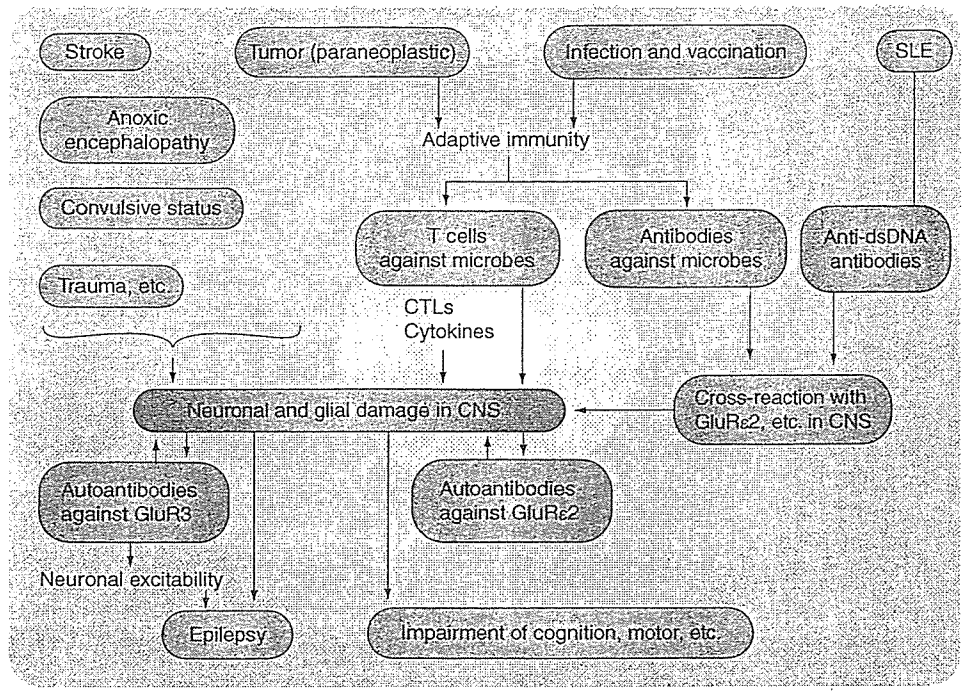
impairment of consciousness, epilepsies, cognitive impairment, and motor disturbance) tend to occur. For instance, while viral infections cause acute viral encephalitis, malignant extracerebral tumors may sometimes also cause paraneoplastic acute or subacute encephalitis. Anoxic encephalopathy in neonatal periods causes West syndrome and CNS infections also cause West syndrome as sequelae. It is postulated that autoimmune processes, including autoantibodies against neural molecules, may contribute to the subsequent processes after acute insults to the CNS (stroke, trauma, anoxic encephalopathy and convulsive status etc.), resulting in autoimmune neuronal damages (Figure 6). Autoantibodies against GluR3 and GluRε2 are found also in patients with various neurological diseases [12,36]. Several pathological roles of autoantibodies against GluR3 have been reported, including excitotoxicity [29], complement-dependent cell death [30] and complement-containing membrane attack complex (MAC) formation [31,32], although the induction of currents through GluR was controversial [33,37]. The MAC is composed of several complements and appears to induce functional pores in the cell membrane, leading to depolarization and osmotic lysis of neurons. These data show that autoantibodies can directly cause impairment of neural functions. Further studies are needed for a better understanding of autoimmune processes in neurological diseases.

## Conclusion

Mechanisms leading from infection or vaccination to the development of epilepsy are not fully understood, but the following mechanisms are proposed: direct infection, reactivation of latent infection, parainfectious mechanism, modification of synaptic transmission and triggering mechanism.

In Rasmussen's syndrome, a prototype of parainfectious autoimmune epilepsy, 44% of patients have preceding infections or vaccinations and 8.8% of patients have head trauma as possible causative factors; thus, the important roles of CTLs and autoantibodies against neural molecules are unveiled. Several HLA class I genotypes are found at significantly higher rates in patients with Rasmussen's syndrome, compared with the Japanese population. Motif analyses of HLA-binding peptides in microbial molecules and neural molecules postulated that CTLs activated by infections may cross-react with neuronal molecules due to molecular mimicry and TCR degeneracy. HLA type may affect the clinical phenotypes of autoimmune-mediated epilepsies.

**Figure 6. Hypothesis of common autoimmune processes leading from neuronal damage to neurological sequelae.**




Noninflammatory insults against neurons and glia may cause production of autoantibodies against neuronal molecules (GluRε2 and GluR3 etc.). These autoantibodies, together with membrane attack complexes, may cause neuronal excitability and neuronal death, resulting in the onset of epilepsy or impairment of brain functions.
CTL: Cytotoxic T lymphocyte; ds: Double stranded; GluR: Glutamate receptor; SLE: Systemic lupus erythematosus.

In acute viral encephalitis, GluR autoimmunity may contribute to the onset of localized encephalitis as well as the development of sequelae in widespread encephalitis. Autoantibodies against GluRε2 may have a causal relation with epileptogenesis following widespread encephalitis.

Noninflammatory insults, such as stroke and anoxic encephalopathy, may precipitate the onset of symptomatic epilepsies due to neuronal injuries. Autoimmune mechanisms, including autoantibodies against neuronal molecules and complement MAC, may contribute to these processes.

## Future perspective

Recently, brain inflammation in epilepsy has been highlighted as a common factor in epilepsies [38]. Elucidation of the autoimmune mechanisms and verification of molecular mimicry between microbial and neuronal molecules in Rasmussen's syndrome may facilitate understanding of the autoimmune process, by which infections or vaccinations lead to the development and aggravation of symptomatic epilepsies other than Rasmussen's syndrome. Prevention of epilepsy onset after CNS infections and novel 'pinpoint' immunological treatment of epilepsies after onset may become possible in the future. Antigen-specific immuno-potentiating or -suppressive therapy with altered peptide ligands may become available for the treatment of some symptomatic epilepsies [39]. For the treatment of West syndrome, adrenocorticotropic hormone is currently used, but other specific immunological treatments may be possible after elucidation of its autoimmune mechanisms in the future.

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## Executive summary

### *Introduction*

- Childhood-onset epilepsy may begin after an infection episode or following vaccination, albeit rarely. After the onset of epilepsy, status epilepticus tends to occur under febrile conditions triggered by infections or vaccinations.
- A total of 12% of admitted cases of intractable epilepsy were attributed to CNS infections (acute viral encephalitis and bacterial meningitis etc.).
- The mechanisms by which infections or vaccinations cause epilepsies are not fully understood, but the following mechanisms are possible: direct infection, reactivation of latent infection, parainfectious mechanism, modification of synaptic transmission and triggering mechanism.
- Few data are available on the relationship between vaccination and epilepsy.

### *Rasmussen's syndrome & causative factors (infections & vaccinations)*

- A total of 44% of patients have preceding infections or vaccinations and 8.8% have head trauma as possible causative factors.
- Two patients were encountered who developed Rasmussen's syndrome after vaccination (Japanese encephalitis vaccine and mumps–measles–rubella vaccine).

### *Rasmussen's syndrome & cytotoxic T cells*

- Cytotoxic T lymphocytes (CTLs) may play a primary role in the development of Rasmussen's syndrome.

### *Rasmussen's syndrome & molecular mimicry defined by human leukocyte antigen*

- T-cell receptors (TCRs) on CTLs recognize both human leukocyte antigen (HLA) class I and its binding peptide to induce apoptosis of targeted cells.
- The genotypes of HLA class I were studied in 16 Japanese patients with Rasmussen's syndrome (9 with epilepsia partialis continua, and 7 without epilepsia partialis continua). HLA-A*2402, HLA-A*0201, HLA-A*2601, HLA-B*5201 and HLA-B*4601 were found at significantly higher rates in the patients compared with the healthy Japanese population.
- Motif analyses of HLA-binding peptides in microbial and neural molecules suggest that cytotoxic T cells activated by infections may cross-react with neurons owing to molecular mimicry and TCR degeneracy.
- HLA type may affect the pattern of CNS involvement in CTL activity and subsequent clinical phenotypes in autoimmune-mediated epilepsies.

### *Acute viral encephalitis & autoimmunity*

- Pathological mechanisms of acute viral encephalitis are divided into the primary viral infection mechanism and the secondary parainfectious autoimmune mechanism.
- Clinically, acute encephalitis is divided into two categories: localized encephalitis and widespread encephalitis. In the initial stage, patients with localized encephalitis showed psychic symptoms, solitary seizures and/or very mild impairment of consciousness. Patients with widespread encephalitis showed profound loss of consciousness and/or convulsive status.
- In patients with localized encephalitis, immunoglobulin (Ig)M autoantibodies against glutamate receptor (GluR)ε2 tended to appear in cerebrospinal fluid (CSF) in the acute or recovery stage and may contribute to disease onset.
- In patients with widespread encephalitis, IgM autoantibodies against GluRε2 tended to appear in CSF in the chronic or recovery stage and may contribute to epileptogenesis and mental retardation as sequelae.

### *Hypothesis of common autoimmune processes leading from neuronal damage to neurological sequelae*

- The author postulates that autoimmune processes, including autoantibodies against neuronal molecules and membrane attack complex, may contribute to the development of sequelae following acute insults (both inflammatory and noninflammatory) to the CNS, including the development of epilepsy.

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Short note

# An improved method for Southern DNA and Northern RNA blotting using a Mupid®-2 Mini-Gel electrophoresis unit

Hirokazu Furuya *, Takeshi Yamada, Koji Ikezoe, Yasumasa Ohyagi, Yasuyuki Fukumaki, Naoki Fujii

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**Abstract**

An improved method for Southern DNA and Northern RNA blotting using the Mupid®-2 Mini-Gel System is described. We get sharp and clear bands in Southern and Northern blotting after only 30 min short gel electrophoresis instead of the several hours large gel electrophoresis of conventional methods. The high electrical voltage with a pulse-like current of the Mupid®-2 Mini-Gel System also allows reduction of the amount of formaldehyde, a harmful reagent, from the gel running buffer in RNA blotting. This minor modification of DNA and RNA blotting technique enables us to perform the complete experimental procedure more quickly economically in less space, than conventional Southern and Northern blotting, as well as using an extremely small amount of formaldehyde in RNA blotting.


*Keywords:* Mupid®-2 system; Northern blotting; Southern blotting; Formaldehyde; Pulse-like electrical current

Polymerase chain reaction (PCR) is the most widely used laboratory technique in molecular biology, and the recent progress in direct PCR-based genome sequencing and quantitative RT-PCR technology, has largely eliminated traditional gene analysis methods, including Southern DNA and Northern RNA blotting.

On the other hand, a difference in amplification speed has become apparent between the genomic regions or genes that are easy to amplify with PCR and those that are hard to amplify. The latter include candidate areas for triplet repeat diseases with expansions that are longer than 0.25 to 6.0 kb,

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such as segments with simple 'CTG' triplet repeats (MIM 160900; myotonic dystrophy type 1 (DM1) [1]), decreased repeats with 10-kb *Eco*RI fragments (MIM 158900; FSH-type muscular dystrophy) [2], 'ATTCT' repeat expansions ranging from 920 to 4140 repeats (MIM603516; spinocerebellar atrophy 10 (SCA10)) [3]), and expanded 'CCTG' repeats ranging from 75 to approximately 11,000 repeats (MIM 602668; myotonic dystrophy type 2 (DM2)) [4], all of which are beyond the capacity of *Taq* polymerase in PCR reaction. Furthermore, expansion of a tandem repeat of a dodecamer ('CC CCGCCCCGCG') in the 5-prime untranslated region of the cystatin B gene (MIM 601145; CSTB) may alter the complex secondary structure of a single strand of DNA in gene transcription, resulting in failure of PCR amplification in Unverricht–Lundborg disease (MIM 254800; ULD) [5]. Thus, for the molecular diagnosis of these neuromuscular disorders, common PCR technology is useless. On the other hand, Northern blotting is still a popular method for analysis of gene expressions in which the splicing pattern is unclear. In such cases, conventional DNA and RNA blotting methods, which are time-consuming and costly, are applied for the diagnosis or analysis of gene expression.

The Mupid®-2 Mini-Gel Electrophoresis Unit (Advance Co., Ltd.) (13.0 cm × 18.0 cm × 5.0 cm in height) is a mini-gel gene analysis system commonly used for preliminary gene analysis or small-scale DNA analysis. Unlike other gel analysis system, it uses a semi-direct pulse-like current instead of the usual power unit, and has a very small gel system (6.0 cm × 5.5 cm, 6 lanes) combined with the power supply in one unit [6,7].

Here we describe a minor modification of DNA and RNA gel electrophoresis using the Mupid®-2 Mini-Gel System that enables us to improve quantification even in a small gel format and to reduce analysis time while decreasing the amount of harmful formaldehyde necessary in RNA gel electrophoresis.

## 1. Southern blotting

Genomic DNA is obtained from human blood samples. Genomic DNA (10 μg) is digested with the restriction enzyme and precipitated with ethanol, and the concentration is quantified by means of a NanoDrop spectrophotometer (NanoDrop Tech). Then 7.5 μg of the DNA is electrophoresed in an agarose gel, using the usual TAE buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.0)) [8,9], in the larger lane of the Mupid®-2 Mini-Gel System and running the gel at 100 V for 30–40 min until the bromphenol blue dye has migrated to the appropriate position. We change the TAE buffer in the

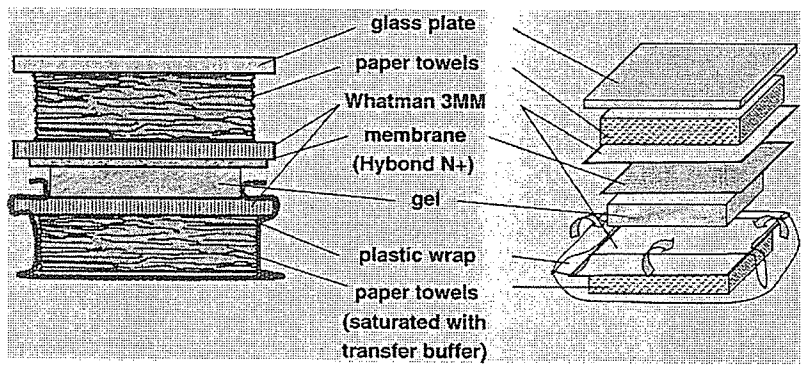


Fig. 1. Disposable gel transfer setup for Southern and Northern blotting via capillary transfer. Paper towels saturated with transfer buffer and covered by plastic wrap is used instead of the reservoir bath, which saves space.

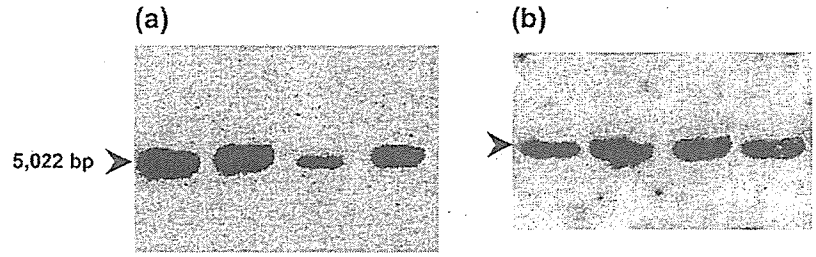


Fig. 2. Southern blot analysis after autoradiography with (a) conventional DNA gel electrophoresis (gel size is 5.5 cm × 14 cm with 6 lanes) and (b) the Mupid®-2 system (5.5 cm × 6.0 cm with 6 lanes). Genomic DNA was digested with *Eco*RI and *Hin*dIII followed by gel electrophoresis. In conventional analysis, 5 h electrophoresis is applied (a). In the Mupid®-2 system, 100 V was applied to a mini-agarose gel for 30 min (b). After transferring DNA, these membranes were hybridized with a $^{32}$P-labeled DNA probe (subcloned exon 2 and 3 genomic DNA fragment of human CSTB gene). Note the sharpness and good isolation of bands in the short gel of the Mupid®-2 system (b).

middle of the electrophoresis if the gel is run more than 30 min to avoid raising the temperature of the buffer. The DNA from this gel is transferred to a Hybond N$^+$ membrane (Amersham Inc.) by the disposable gel transfer system (Fig. 1) using an alkaline denaturing solution (0.5 M NaOH, 1.5 M NaCl) as a transfer buffer. These membranes are hybridized with a DNA probe and labeled by the Random Primer labeling system using [$^{32}$P]dCTP as the radioactive nucleotide. Pre-hybridization and hybridization are performed using Church's hybridization buffer without bovine serum albumin (BSA) [9]. Blots are exposed on imaging plates, and the radioactivity of the bands is visualized with the BAS2000 System (Fujifilm Inc.) (Fig. 2).

## 2. Northern blotting

Total RNA is extracted from the cultured cells with an RNeasy Mini kit (Qiagen), and the concentration is measured by means of a NanoDrop spectrophotometer (NanoDrop Tech). Then 1 to 10 μg of the total RNA is electrophoresed in a 1% agarose gel using the usual TAE buffer without formaldehyde in the large well of the Mupid®-2 Mini-Gel System. Total RNA samples are prepared for loading with 1 × formaldehyde gel-running buffer (17.5% formaldehyde and 50% formamide in

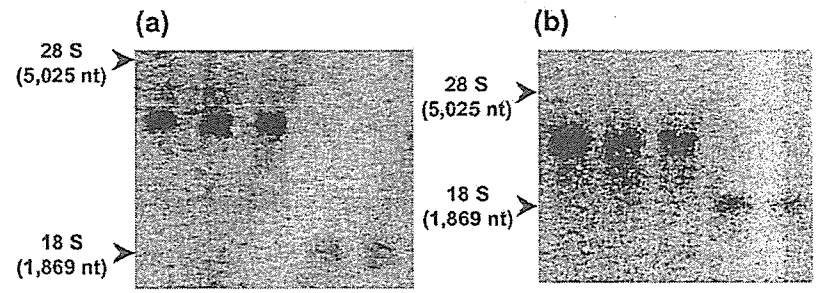


Fig. 3. Northern blot analysis after autoradiography with (a) conventional RNA gel electrophoresis and (b) the Mupid®-2 system of total RNA extracted from PC12 cell stably expressing the luciferase gene [10]. In conventional analysis, 30 V was applied to 1% agarose gel with formaldehyde for 5 h electrophoresis (a). In the Mupid®-2 system, 100 V was applied to a mini-agarose gel without formaldehyde for 30 min (b). After transferring RNA to the Hybond N$^+$ membrane, these membranes were hybridized with a $^{32}$P-labeled luciferase cDNA probe. The gel size is the same as that of Fig. 2.

the final concentration) followed by vortexing, microcentrifuging briefly, and incubating 5 min at 65 °C [8]. A 10% volume of the formaldehyde gel-loading buffer is added, and the gel is run at 100 V for 30–45 min until the bromphenol blue dye has migrated to the appropriate position [8]. We change the TAE buffer in the middle of the electrophoresis if the gel is run more than 30 min. Then, the RNA in the gel is transferred to a Hybond $N^+$ membrane by the disposable gel transfer system (Fig. 1) with 20 × SSC as a transfer buffer. Pre- and hybridization and visualization of the target band are performed by the same method used for Southern blotting analysis [8,9] (Fig. 3). The radioactivity of the mRNA bands demonstrates the gene expression quantitatively [10].

In both Southern and Northern blotting, we get a band that is as clear and sharp in a short gel (6.0 cm in length) as in a 14.0 cm gel (Figs. 2, 3). This is probably due to the character of the semi-direct electrical current in the Mupid®-2 system. Nucleotide fragments form a strong secondary structure, especially in RNA, because RNAs are single-stranded and able to form secondary structures by intramolecular base pairing. RNAs must therefore be electrophoresed under denaturing conditions if good separations are to be obtained.

This is the reason that same-size nucleotides do not necessarily migrate exactly the same distance. This phenomenon is widely exploited in the analysis of single strand conformation polymorphisms to detect RFLP. To avoid this problem, especially in analysis of chromosome separation, pulse-field gel electrophoresis is usually applied. Because the Mupid®-2 system generates a pulse-like electrical current and runs the gel at a high voltage in a short running time, we get a sharp band.

Denaturation is achieved either by adding formaldehyde to the gel and loading buffers or by treating the RNA with glyoxal and DMSO prior to loading for conventional Northern blotting. However, our protocol using the Mupid®-2 system eliminates the need for formaldehyde in the running gel buffer. Since the Mupid®-2 system generates a pulse-like electrical current and runs the gel at a high voltage in a short time, we get a sharp band even in the formaldehyde-free agarose gel and running buffer [6,7].

In conclusion, a minor modification of Southern and Northern blotting using the Mupid®-2 system allows us to quantify gene expression quickly, save space, and reduce costs, while using an extremely small amount of formaldehyde in RNA blotting.

## Acknowledgements

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# Epidemiology of Severe Cutaneous Adverse Drug Reactions in Japan

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# Epidemiology of Severe Cutaneous Adverse Drug Reactions in Japan

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**Abstract** **Adverse drug reactions (ADR) such as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS) are potentially fatal disorders. To define the clinical features of these diseases in recent years, a summary of reported cases of SJS (44 cases), TEN (51 cases), and DIHS (118 cases) is shown. The drugs commonly identified as etiological agents of SJS and TEN were nonsteroidal anti-inflammatory drugs, antibiotics, and anticonvulsants. The mortality rates were 9.8% (TEN) and 2.3% (SJS). These are lower than the previous reports in Japan. In the patients with DIHS, 63.7% of the causative drugs were anticonvulsants, especially carbamazepine. Multi-organ involvement including hepatitis, myocarditis, type I diabetes, and encephalitis was observed. Reactivation of HHV-6 was detected in 83.9% of patients.**

**On the other hand, reactivation of cytomegalovirus was observed in 4 patients without HHV-6 reactivation. Therefore, cytomegalovirus might be involved in at least some cases of DIHS without HHV-6 reactivation.**

***Key words : drug-induced hypersensitivity syndrome, epidemiology, Stevens-Johnson syndrome, toxic epidermal necrolysis***

## はじめに

重症薬疹は，その発症頻度が低いことから個々の施設で診療する機会は少なく，そのため同一施設において統計的検討を行うことは困難である。しかしその社会的重要性からこれまでに多くの症例報告がなされ，それらを解析することにより最近の重症薬疹のありようを明らかにすることは可能と思われる。

重症薬疹の代表としてあげられる Stevens-Johnson 症候群（SJS）および中毒性表皮壊死症（toxic epidermal necrolysis; TEN）に加え(1)，近年本邦では薬剤性過敏症症候群（drug-

induced hypersensitivity syndrome: DIHS）が重症薬疹の独立した一型として認識されている(2-4)。これらはいずれも高熱を伴って発症し，肝障害や多臓器障害を合併してときに死に至る。DIHSでは多くの症例でその経過中にヒトヘルペスウイルス-6（humanherpes virus-6: HHV-6）の再活性化がみられることから，薬疹の重症化とウイルス感染の関係が注目されている。

本稿では，本邦におけるSJS, TEN, およびDIHSの最近の報告例の集計結果からこれらの疾患の現状を示し，さらにSJS, TENではそれらを過去の報告例を比較することによりその動向について概説してみたい。

## 1　SJS, TENの最近と過去の報告例の比較

1999年10月から2005年10月までの6年間に論文として報告された本邦のTEN 51例，SJS 44例を最近の症例としてまとめ，過去（1981年～1997年）の報告例（TEN 287例，SJS 269例）(5, 6)との異同を検証すると以下のようになる。

1．年齢，性別：最近の症例における発症年齢は幼児から80歳代におよび，平均年齢はいずれも45歳であった（Table 1）。過去の報告例では平均年齢はTENで44歳，SJSで33歳であり，SJSで近年平均年齢の上昇がみられる。男女比はTENではほぼ同数であるものの，SJSでは近年1：1.82と，過去の報告（1：1.06）と比較して女性例が多く報告されている。以前よりSJSにおいて小児では男児が，成人では女性が多いことが指摘されており(7)，成人例が増加したことが女性例の増加の原因と推測される。

2．死亡率：最近の報告例における死亡率はSJSで2.3%，TENで9.8%であり（Table 1），過去の死亡率（TEN 21.6%, SJS 6.3%）と比較すると低くなっている。これは，発表症例の選択にあたりバイアスがかかってはいるものの，後述する薬疹治療の進歩が死亡率を低下させていることを示唆するものと思われる。

3．臨床型：SJSは多形紅斑と結膜や口唇口腔などの壊死性の粘膜疹を認め，水疱形成・表皮剥離が拡大するとTENに進展する(1)。TENは，全身の紅斑と広範な表皮剥離を認め，SJSから進展したもの（TEN with spots）のほか，びまん性紅斑から急激に表皮剥離をきたすもの（TEN without spots），固定薬疹から進展したものなどがある(1)。SJSとTENは近年（Fig. 1）および過去の報告において，ほぼ同数報告されており，TENの多くはSJSから進展したものである。また，多発性固定薬疹の報告はみられるものの，TENに至ったという報告は近年みられない。なお，報告者の記載に合わせて表皮剥離面積が体表面の10%～30%未満をSJS/TEN overlapとしてFig. 1に示したが，その重症度から他の図表ではこれらもTENとして扱った。

4．発症原因：近年の報告ではTENのすべての症例で原因は薬剤とされている。SJSでは薬剤が原因とされたものは全体の70%であり，過去の報告例（62%）と比較して，その割合に大きな変化はみられない。薬剤以外の原因は感染症または感染症か薬剤か明らかでないとされている。感染症では*Mycoplasma pneumoniae*（*M. pneumoniae*）が最も多く，*M. pneumoniae*感染がみられた4例のうち3例は15歳以下であった。これは*M. pneumoniae*感染が若年者に多くみられ，それによるSJSも小児に多いというこれまでの報告と一致する(8)。

原因薬剤は，消炎鎮痛解熱薬（感冒薬を含む），抗菌薬，抗痙攣薬が多くを占め，次にアロプリノールが位置する。これを過去の報告例と比較すると，前2者の割合はほとんど変らないものの，抗痙攣薬の割合が若干増加している（Fig. 2）。なお，

Table 1.　Analyzed cases of TEN, SJS and DIHS

| | Age, years (mean) | Male/Female | Dead cases, No (%) |
|---|---|---|---|
| TEN　(n= 51) | 1- 88 (45.8) | 1 : 1.04 | 5 (9.8) |
| SJS　(n= 44) | 4- 84 (45.0) | 1 : 1.82 | 1 (2.3) |
| DIHS　(n=118) | 0- 89 (48.6) | 1 : 0.8 | 4 (3.4) |

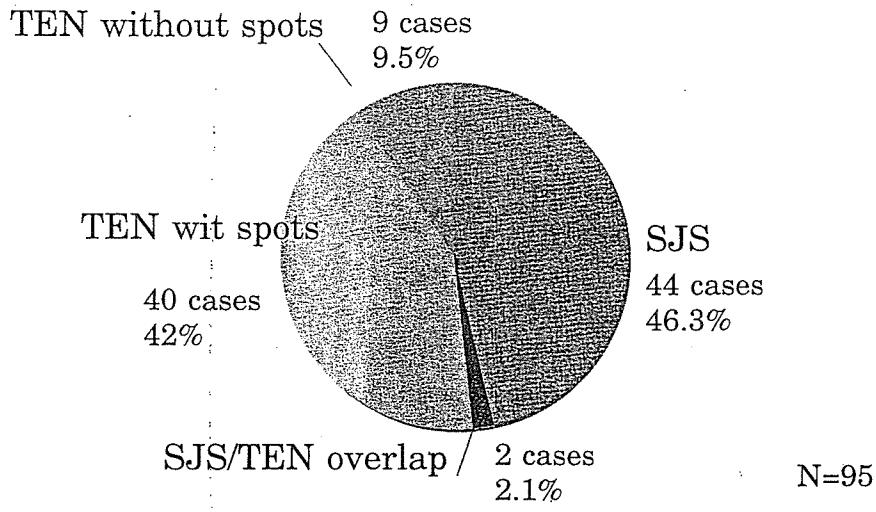


Fig. 1　Clinical types of SJS and TEN
The clinical classification was based on the type and distribution of the skin lesions and the percentage of body surface area involved in blisters and erosions (Bastuji-Garins S et al, Arch Dermatol 129: 92-96, 1993).

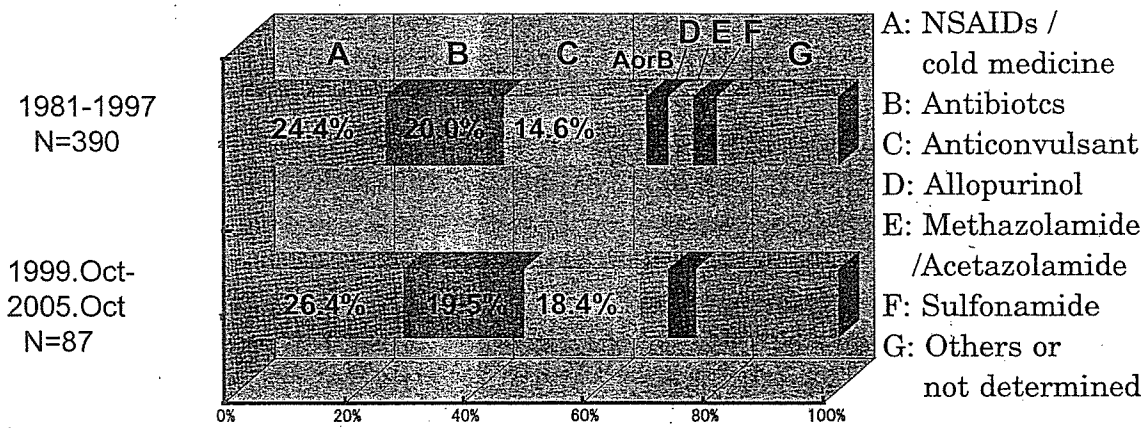


Fig. 2　Causative drugs in patients with SJS and TEN －Comparison between the data of 1981-1997 and 1999 Oct-2005 Oct

過去にSJS, TENの発症が多くみられた緑内障治療薬のメタゾラミドは本邦では1999年12月以降販売中止となっており，近年ではそれによる発症はみられない。

5．治療：ステロイド薬の全身投与は本邦では2000年以前も大部分の報告例で行われていたが，パルス療法は一部の施設でしか行われていなかった。しかし近年ではパルス療法が行われる比率が増加し，ステロイド量の記載のある65例のうち50.8%にパルス療法が施行されていた。さらに血漿交換療法の併用例の増加や過剰な免疫反応の調整を目的とした大量ヒトガンマグログリン投与(9)を行うことにより救命しえたという報告が2000年以降みられるが，その数は多いものではない。

## 2　DIHSの現状とSJS, TENとの比較

DIHSは1998年に本邦からHHV-6の再活性化を伴う重症薬疹として報告されて以来(2, 3)，こ

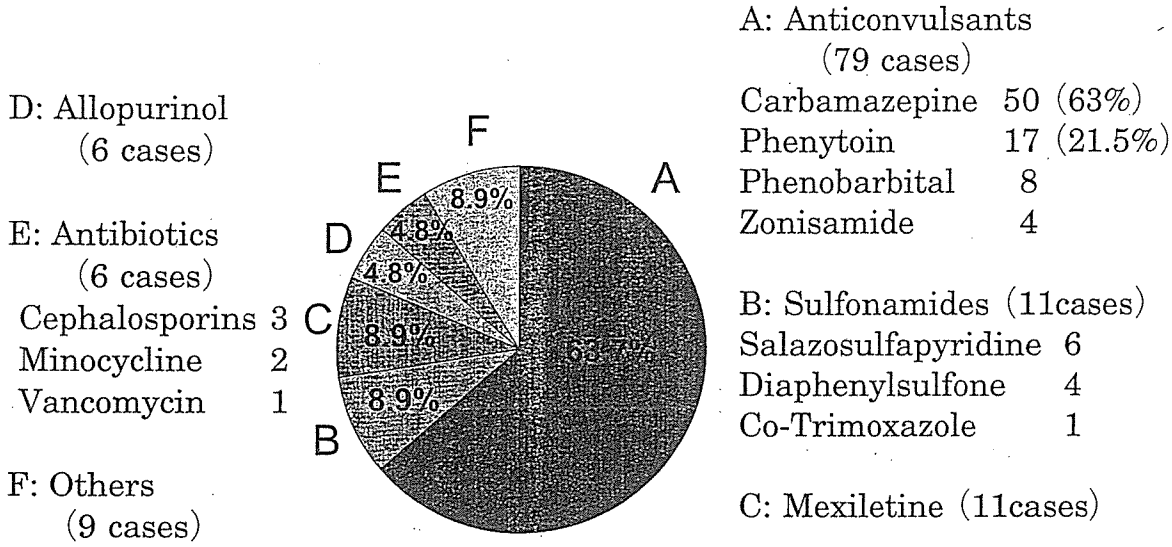


Fig. 3　Causative drugs in patients with DIHS

れまでに多くの報告がなされている。その臨床的特徴は，薬剤投与から発症までの期間が他の薬疹より長いこと，原因薬剤が極めて限られること，高熱を伴う全身性の紅斑を認め，薬剤中止後も症状は進行し，リンパ節腫脹や肝機能障害をはじめとする臓器障害がみられることなどがあげられる。さらに，末梢血検査では，著しい白血球の増加，好酸球や異形リンパ球の増加がみられる(4, 10)。経過中の HHV-6 再活性化は特異的抗体価の上昇や HHV-6DNA の検出により証明されるが，cytomegalovirus (CMV)，HHV-7 など，HHV-6 以外のヒトヘルペスウイルスの活性化についても報告されている(11, 12)。

これまでにわれわれは上記臨床所見や検査結果について十分な記載がある症例報告についての解析結果を報告してきた(13)。ここではあらたに 2004 年 10 月までに報告された症例を加えた 118 例の報告（HHV-6 の再活性化の有無について検討されているもののみ集計）を以下に示し，近年の SJS，TEN 症例との疫学的違いとその解釈について述べる。

1．年齢，性別，死亡率：DIHS 報告例の平均年齢は 48 歳で TEN, SJS とほぼ等しく，性別ではわずかに男性が多い（Table 1)。これらの報告における死亡例は 4 例（死亡率 3.4%）であったが，実際には臨床症状から DIHS が疑われるものの HHV-6 や，他の humanherpes virus の再活性化の有無について検討されていない学会発表症例に死亡例がみられる。このことから，本邦における DIHS の死亡例はより多いものと思われる。

2．原因薬剤と投与から発症までの期間：DIHS の原因薬剤は抗痙攣薬が 63.7%を占め，特にカルバマゼピンが多く，フェニトイン，フェノバルビタールと続く（Fig. 3)。抗痙攣薬の他にはサルファ剤，メキシレチン，アロプリノールの報告が多い。その他の薬剤で HHV-6 の活性化を伴った DIHS と診断されたものは，ミノサイクリン，抗結核薬，炭酸リチウム，シアナマイド(14)，などがあり，臨床症状から DIHS が強く疑われたものの HHV-6 の活性化が証明されなかったものとしてセフェム系抗菌薬などがある。

薬剤投与から症状の出現までに要した期間は DIHS では TEN や SJS に比較すると長いことが知られている。多くは 3 週間から 5 週間後に発症するが，なかには 8 週間以上経過してから発症した症例もみられる（Fig. 4)。

原因薬剤が限られることと長期間の投与後に発症するということは，DIHS の発症には SJS, TEN と異なり単に薬剤に対する感作が成立するだけでなく，免疫変調作用を有する限られた薬剤が，長期間投与されることが必要であることを示すものと思われる。すなわち，投与された薬剤が

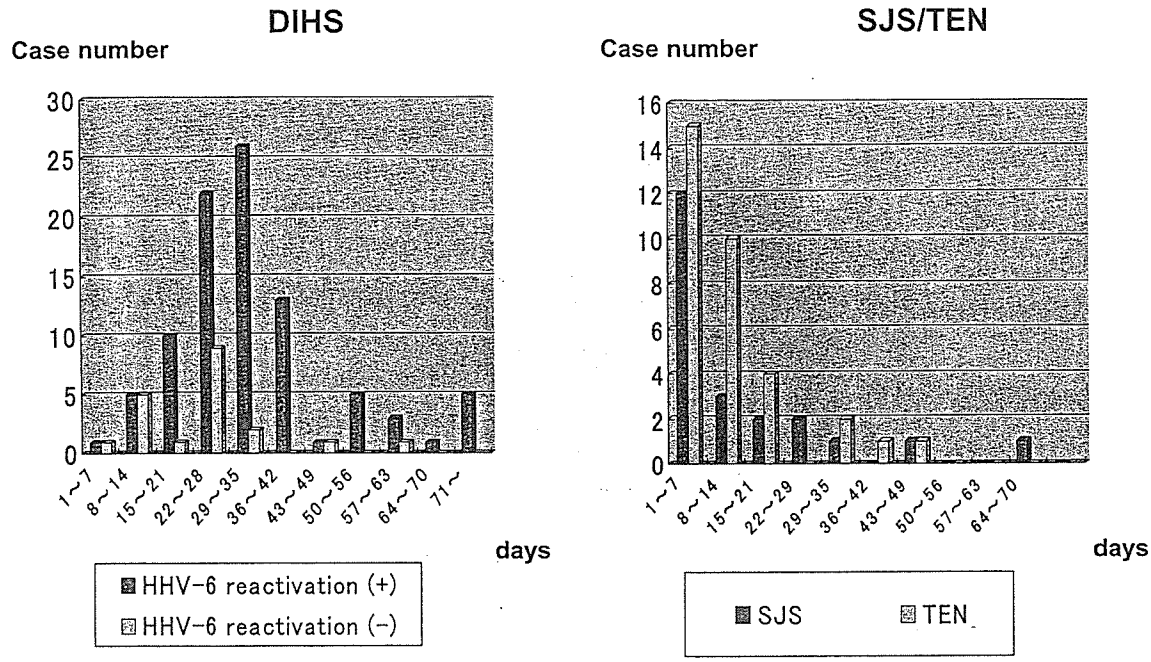


Fig. 4 Time between the first drug intake and the onset of symptoms in patients with SJS/TEN and DIHS
DIHS: 1998-2005 Oct, TEN, SJS: 1999 Oct-2005 Oct

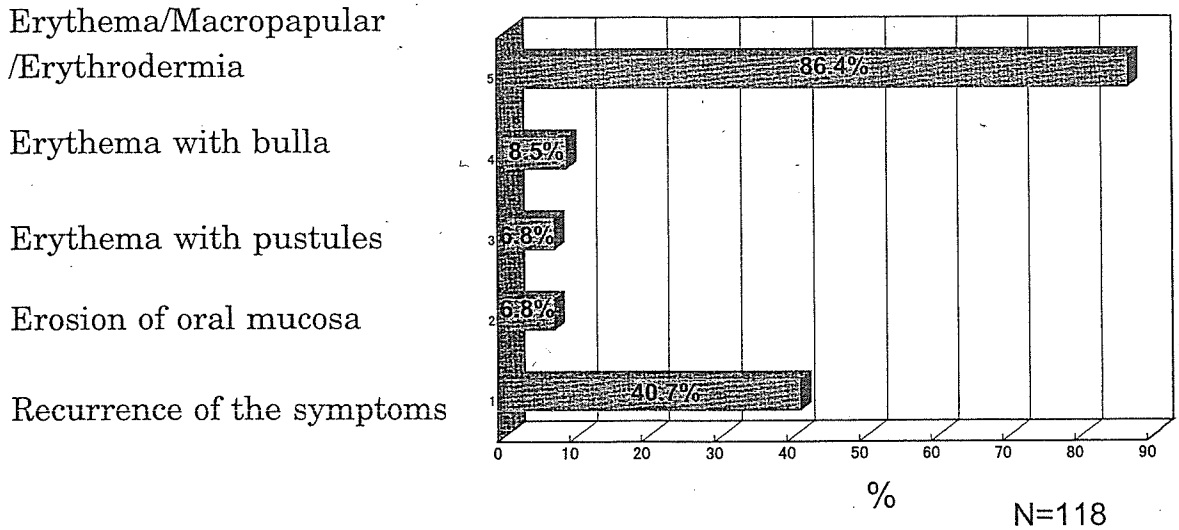


Fig. 5 Eruptions and recurrence of the symptoms of DIHS

HHV-6 の再活性化やそれに対する過度な免疫反応を引き起こすような免疫変調を誘導することがその発症に重要であることを示唆するものと考えられる。免疫変調を示す 1 例としては，抗痙攣薬による DIHS において，発症時に免疫グロブリンの低下を認め，治癒後には正常値に復していることが報告されている(15, 16)。

3．皮疹および経過：DIHS の代表的皮疹としては顔面腫脹を伴う全身の紅斑丘疹とさらに進展した紅皮症が知られているが，症例によっては水疱や膿疱の形成をみる（Fig. 5)。水疱は口囲や四肢の紅斑上に小水疱が多発することが多く，病理