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**備考：**

この研究は熱帯医学の分野における登載登載が行われた研究論文を対象としています。
Serotype Identification of Dengue Virus Isolates by an Enzyme-linked Immunosorbent Assay Employing a Protein Denaturing Agent.

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Abstract: Serotypes of dengue isolates were identified successfully by an enzyme-linked immunosorbent assay (ELISA) reacting with crude viral antigens and conventional hyperimmunized anti-dengue mouse antibodies. In the ELISA, a protein denaturing agent, sodium thiocyanate (NaSCN) was employed to discriminate between serotype specific reaction and nonspecific cross-reaction among four serotypes of the virus. Antigen fractions in sucrose gradient were highly cross-reactive to both homologous and heterologous antibodies in the absence of NaSCN in the ELISA, and their serotype specificities were improved by the treatment of NaSCN in the ELISA. Fifteen out of 38 strains (ca. 38%) and 12 out of 38 strains (ca. 31.5%) of dengue isolates were recovered and identified by IFA staining and the ELISA from field specimens kept at -80°C for 7 years, respectively.

Key words: Dengue isolates, serotyping ELISA

INTRODUCTION

Dengue virus, family Flaviviridae, genus flavivirus, consists of four closely related serotypes, and causes dengue and dengue hemorrhagic fever in man. Serotype identification of dengue isolates is not easy because of the extensive serological cross-reaction among four serotypes of the virus. So far, many techniques have been developed, i.e. inoculation into suckling mouse brain (11), into the thorax of Aedes (14) or Toxorhynchites (20) mosquitoes, or into vertebrate (7) or mosquito (23) cell cultures; for serotyping, complement fixation (CF) test (21), immune adherence hemagglutination (9), IFA staining with monoclonal antibodies (6) and ELISA with monoclonal (12) or conventional mouse antibodies (16, 17).

At present, it is well known that the use of serotype specific monoclonal antibodies gives rapid and reliable data for us, however the adoption of these monoclonal antibodies is not simple and is hampered by the circumstances of virus laboratories there, e.g. the lack of equipment and skilled personnel, high cost and long periods to establish specific hybridoma cell clones and the maintenance of them. Above situations are particularly seen in the laboratories of developing countries where dengue viruses are prevalent.

In this regard, the author previously developed an ELISA for serotyping of prototype dengue viruses adopting crude viral antigens and conventional mouse antibodies (18). In that
assay, a protein denaturing agent, 0.5M NaSCN was used to make the stringent condition for the control of nonspecific cross-reactions in heterologous combinations of mouse antibodies and viruses. In this report, the author reports the results obtained with the practical application of the NaSCN-treated ELISA for serotyping of dengue isolates.

**MATERIALS AND METHODS**

**Cells and Viruses:** C6/36 cells (8) were subcultured in 10% FCS-Eagle's MEM supplemented with 0.2mM non-essential amino acids at 28°C. Twelve strains of field isolated dengue viruses: type 1, No. 12, V-6 and D183 strains; type 2, SI-6, SI-53 and CH229A strains; type 3, No.16, D224 and DD9 strains and type 4, No.15, No.17 and D80 strains were inoculated in C6/36 cells, and maintained in the same medium but reduced FCS at 2% for 4 to 5 days at 28°C. Infected cells were separated from culture fluids and washed twice with phosphate buffered saline and fixed with acetone and served for the standard IFA staining. Infected cell culture fluids were clarified from cell debris at low speed centrifugation, and then stored at -80°C before use.

**Antibodies and Assays:** Anti-dengue mouse immunoglobulin G (IgG) for four standard strains of dengue virus (Hawaii, New Guinea C, H87 and H-241) were prepared from ascitic fluids of mice immunized with viral antigen (8) extracted from infected suckling mouse brains by using sucrose and acetone (3). For the preparation of high-titered antibody, mice were immunized more than five times with the antigen according to the method described previously (2). IgG was purified from ascitic fluids of mice by ammonium sulfate precipitation and DEAE-cellulose column chromatography. A part of the IgG was biotinylated with N-hydroxysuccinimidobiotin according to the method described previously (16), and another part was saved for the coating antibody to capture viral antigens on the solid phase. The ELISA method described previously was followed (18). Briefly, microplate wells were coated with anti-dengue mouse IgG at a concentration of 10 µg per 0.1 ml in 0.05M carbonate buffer (pH 9.6). Undiluted infected cell culture fluids as the crude viral antigen were reacted to antibody-sensitized wells at 4°C for overnight. Then, antigen captured wells were reacted to biotinylated antibodies with and without 0.5M NaSCN in the serum diluent and incubated at room temperature for 2 hr. Antigen-antibody complexes were detected with streptavidin-conjugated horseradish peroxidase, and color was developed with o-phenylenediamine dihydrochloride and H₂O₂ in citric acid-phosphate buffer (pH 5.0), and absorbance at 492 nm was measured by an ELISA reader.

Serotype specific monoclonal antibodies, 15F3, 3H5, 5D4 and 1H10 for types 1, 2, 3 and 4 were provided by Dr. N. Karabatsos, CDC, U.S.A. and used for the detection of specific viral antigens in infected C6/36 cells according to the standard IFA technique using FITC conjugated anti-mouse IgG goat antibody (6).

**Sedimentation analysis of viral antigens:** A type 2 dengue virus, SI-6 strain, infected C6/36 cell culture fluid (500ml) was harvested on the 5th day of infection at 28°C. Viral antigens in the culture fluid were concentrated by 50% saturation with ammonium sulfate and
centrifuged at 10,000xg for 30 min at 4°C, the pellet was suspended in borate buffered saline pH9.0 (BS 9.0) containing 0.4% bovine serum albumin and the resultant suspension (0.05 of original volume) was centrifuged at 105,000xg for 2.5 hr at 4°C, the pellet was resuspended with small volume of BS 9.0 (0.001 of original volume) and further fractionated through a linear sucrose gradient (5–25%) at 90,000xg for 3 hr at 4°C. Fractions (ca. 0.3ml) were collected and assayed HA (4), CF and ELISA activities.

RESULTS

Fig. 1 showed the value of antibody dissociation after the treatment of 0.5M NaSCN in the serum diluent. Viral antigens in infected cell culture fluids were assayed for their antigenic reactivities using both homologous and heterologous antibodies with and without 0.5M NaSCN in the ELISA.

In the preliminary experiments, antibody titrations were set to discriminate between homologous and heterologous reactions using prototype dengue viruses. Titration procedures showed the major advantage that the titer or the serum dilution was directly indicative of the antibody activity, also the antibody dose—response curve usually showed a sigmoid form. A problem was that the discrimination level cut the dose—response curve in the tail—part of the sigmoidal curve and, consequently, low accuracy was obtained. In this regard, assays were tried to avoid this problem by measuring the antibody curves at highest sensitivities, i.e. in the straight part of the curve and to compare the absorbance in the presence and absence of 0.5M NaSCN in the serum diluent (data not shown).

As shown in the figure, the decrease in homologous reaction was generally lower than that in heterologous reactions, in other words the serotype specific reaction was directly indicative of the lower value of antibody dissociation after NaSCN treatment in the ELISA.

The data suggested that the procedures not only distinguished serotypes of prototype dengue viruses, but also distinguished serotypes of dengue isolates.

Sedimentation profiles of viral antigens were studied (Fig. 2). Antigenic reactivities were detected at two peaks: one at higher concentration of sucrose gradient and HA reactive and another at lower concentration of sucrose gradient and CF reactive which represented RHA and SCF deduced from their sedimentation characteristics in the published data (22). Fractions in sucrose gradient were comparatively titrated for anti—type 1 and —type 2 antibodies in the ELISA. As shown in the figure, fractions were highly cross—reactive to both type 1 and type 2 antibodies in the absence of NaSCN in the ELISA, and their serotype specificities were improved by the treatment with NaSCN in the ELISA.

To assess the feasibility of the NaSCN—treated ELISA in diagnostic purpose, 38 strains of dengue isolates were used in the study (Table 1). Dengue isolates, type 1, 9 strains; type 2, 20 strains and type 3, 9 strains were isolated and identified in the Philippines in 1987, and then imported into Japan and kept at −80°C for 7 years until 1994 (17). Specimens as the form of infected C6/36 cell culture fluids were inoculated in C6/36 cells and incubated at 28°C for 4 to 5 days. Viral antigens in infected cells and culture fluids were
Fig. 1. Value of antibody dissociation after NaSCN treatment in the ELISA

Twelve strains of dengue isolates were comparatively identified by using IFA staining according to the method described in the text. Viral antigens in infected culture fluids were reacted to both homologous and heterologous antibodies with and without 0.5M NaSCN in the antibody diluent. Remaining absorbance (O.D. obtained in the absence of NaSCN—O.D. obtained in the presence of NaSCN) was counted as the value of antibody dissociation.
Fig. 2. Distribution of viral antigens in sucrose gradient
Fractions in sucrose gradient were assayed HA, CF and ELISA activities. HA and CF titrations were carried out following the method described elsewhere. Fractions (1:1) were comparatively reacted to biotinylated anti-type 1 (▲, △) and type-2 (●, ○) antibodies at a 1:64 dilution with and without 0.5M NaSCN, indicated as broken and solid lines, respectively.

Table 1. Detection of viral antigens from field specimens by using IFA and ELISA
Thirty eight strains of dengue isolates kept at −80°C for 7 years were recovered and infected in C6/36 cell cultures. Viral antigens in infected cells and culture fluids were detected separately by IFA staining and the ELISA described in the section, Materials and Methods.

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<th>Assayed by</th>
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<td>(+)/(+)</td>
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<tr>
<td>IFA</td>
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detected separately by using IFA staining and the ELISA. Fifteen strains of the virus: type 1, 2 strains; type 2, 12 strains and type 3, 1 strain were recovered and identified by IFA staining in infected cells, and 12 antigen positive culture fluids: type 1, 1 strain; type 2, 10 strains and type 3, 1 strain were detected in the ELISA. Three IFA positive cases, each 1 case in types 1, 2 and 3 were not detected for their specific viral antigens in culture fluids by the ELISA. Finally, 15 out of 38 cases (ca. 39%) and 12 out of 38 cases (ca. 31.5%) were detected for their specific viral antigens in IFA staining and the ELISA, respectively.

DISCUSSION

The results suggested that the employment of a protein denaturing agent, NaSCN, in the reaction mixture of dengue viral antigen and conventional anti—dengue mouse IgG was useful for the serotype identification of dengue isolates in the ELISA.

In the previous report, the author suggested a possibility on the serotyping of dengue viruses using prototype strains and mouse antibodies (18). The author confirmed the feasibility of this method on the practical application for dengue virus identification in diagnostic purpose.

In this method, NaSCN was employed for making stringent conditions for antigen—antibody reactions in the ELISA so that the binding of nonspecific cross—reactive antibody was inhibited, whereas type specific antibody was still allowed to bind to the antigen, otherwise the reagent probably affected the fine structure of both the antigenic determinants and the reactive sites of antibody. Actually, NaSCN has been employed for the elution of antibodies or antigens in immunoaffinity chromatography and has been known to cause reversible denaturation of protein structure (1, 5). On the other hand, it is well known that the affinity or avidity of antibody increases progressively with time after immunization of animals (19). Inouye et al. (10) demonstrated successfully the difference of antibody avidity in human sera between recent and remote infections of Japanese encephalitis, rubella and rota viruses using the ELISA treated with guanidine hydrochloride. In the present study, the NaSCN treatment in ELISA might introduce the similar mechanisms that were reported previously (10, 15). The author adopted hyperimmunized mouse antibodies which were collected later than 60 days after the first immunization and estimated as high affinity antibodies produced at late stage of immunization.

So far, ELISA has been developed for diagnostic medicine and biological studies (13). The procedures, which are simple to perform, give an excellent result and take advantages: high specificity of antibodies for a given antigen, powerful amplification of chemical reactions by enzymes, relatively cheap equipment is required and the assays are feasible in field conditions and may be performed rapidly and easily. The ELISA procedures described here took some advantages: hyperimmunized mouse antibodies were easy to prepare in many virus laboratories equipped with the conventional techniques, biotinylation of the antibody was quite simple compared with the conjugation of the antibody with enzymes and streptavidin conjugated peroxidase was commercially available and stable for more than one year at 4°C.
Although many advantages have been counted for diagnostic or research purposes, problems involving specificity may arise if starting antibody has undesired reactivities. This method might be useful for analysis of antigenic relatedness between closely related viruses. Antibodies in a hyperimmunized serum were considered to react less avidly with heterologous than with homologous viruses under the stringent condition in the ELISA.

**REFERENCES**


