Detection of Dengue 4 Virus Core Protein in the Nucleus. II. Antibody against Dengue 4 Core Protein Produced by a Recombinant Baculovirus Reacts with the Antigen in the Nucleus

By YOSHIHIRO MAKINO,* MASAYUKI TADANO, TOSHIKAZU ANZAI, SHAO-PING MA, SHINOBU YASUDA AND TOSHIHIKO FUKUNAGA
Department of Virology, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-01, Japan

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SUMMARY

The dengue 4 virus (DEN-4) core gene and part of the PreM genes were inserted into the baculovirus polyhedrin gene region. The recombinant baculovirus directed the synthesis of the DEN-4 core protein fused to a part of the polyhedrin protein (Mr 25K), as determined by Western blot analysis using DEN-4 core monoclonal antibody. A mouse polyclonal antibody prepared against the DEN-4 core fusion protein showed antigenic reactivity with the authentic DEN-4 core protein (Mr 15.5K) present in the nucleus as well as in the cytoplasm of DEN-4-infected Vero cells as demonstrated by the peroxidase-antiperoxidase staining method. This antibody did not react with cells infected with DEN-1, -2, -3 or Japanese encephalitis virus, or mock-infected cells.

INTRODUCTION

Dengue (DEN) virus, a member of the Flavivirus genus of the family Flaviviridae, is an enveloped, positive-stranded RNA virus, 40 to 60 nm in diameter and causes a mosquito-transmitted disease affecting mankind in the tropical and subtropical areas of the world. There are four serotypes that can be distinguished by the complement fixation test (Sabin & Young, 1949) and neutralization test (Russell & Nisalak, 1967). Recently, cDNAs of DEN-1, DEN-2 and DEN-4 have been cloned and the complete or partial nucleotide sequences have been reported (Zhao et al., 1986; Mackow et al., 1987; Mason et al., 1987; Hahn et al., 1988; Deubel et al., 1988). The DEN genomic RNA is about 11 000 nucleotides in length (Mackow et al., 1987; Hahn et al., 1988). The protein coding order deduced from the sequence data is 5'-C-PreM, M-E-Ns1-nS2a, nS2b-nS3-nS4a, Ns4b-Ns5-3', which is the same as that of other reported flavivirus coding orders (Rice et al., 1985; Castle et al., 1986; Wengler et al., 1985; Sumiyoshi et al., 1987; Coia et al., 1988).

Recently, we obtained a monoclonal antibody (MAb) to DEN-4 core protein. With the use of this MAb, we observed the accumulation of DEN-4 core antigen in the infected cell nucleus (Tadano et al., 1989). It is generally acknowledged that there is no specific nuclear involvement in flavivirus replication (Westaway, 1980). The migration of DEN core protein into the nucleus has not been reported. Therefore, in the experiments reported here we examined further the presence of intranuclear core antigen in DEN-4-infected cells using antibody to DEN-4 core protein produced by a recombinant baculovirus.

METHODS

Cells. A continuous cell line of Spodoptera frugiperda, SF-9, was obtained from the American Type Culture Collection and grown in TNM-FH medium containing 10% foetal calf serum (FCS) (Summers & Smith, 1987). The cells were used for the propagation of baculoviruses, and for their plaque assays. Vero cells were grown in Eagle's MEM containing 5% FCS, and used for the propagation of DEN viruses and Japanese encephalitis (JE) virus.

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Viruses. The baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV, strain E2) and the plasmid transfer vector (pAc373) were kindly supplied by Dr M. D. Summers (Texas A & M University, College Station, Tx., U.S.A.). AcNPV DNA was prepared as described (Summers & Smith, 1987). Strains of DEN and JE viruses used were as follows: DEN-1 Hawaiian, DEN-2 New Guinea B, DEN-3 H-87, DEN-4 H-241 and JE Nakayama. The stock viruses were prepared as 10% suckling mouse brain homogenates.

Construction of recombinant baculovirus. Cloning of DEN-4 cDNA and its nucleotide sequence have been reported (Zhao et al., 1986). Plasmid DNA containing the gene for the DEN-4 core protein (plasmid F-19; Zhao et al., 1986) was digested with restriction enzymes (BglII and NcoI) to produce a fragment (DEN-4 nucleotide positions 89 to 553) that contained the entire DEN-4 core and part of the PreM region. The fragment was blunted-ended with the DNA polymerase Klenow fragment and the ends were converted to a BamHI site by the addition of a BamHI linker. The fragment was then inserted into the BamHI site of plasmid transfer vector pAc373 downstream from the promoter for the baculovirus polyhedrin gene, and thus the first AUG sequence used as the start site was part of the amino-terminal sequence of the core protein. The transfer vector DNA (2 μg) was cotransfected with AcNPV genomic DNA (1 μg) into Sf-9 cells which were then cultured at 28 °C for 4 days. The culture fluid was harvested and plaque assayed in Sf-9 cells. The recombinant virus resulting from homologous recombination between the plasmid and the viral DNA in the polyhedrin gene region formed occlusion-negative plaques, and was isolated and cloned three times in Sf-9 cells. An outline of the experiment is shown in Fig. 1. The enzymes and linker were purchased from Nippon Gene Company and Takara Shuzo.

Western blot analysis. Sf-9 cells infected with the recombinant virus or AcNPV, or mock-infected, were cultured for 2 to 3 days at 28 °C. The cells were harvested and pelleted by low speed centrifugation. After washing once with phosphate-buffered saline (PBS), the cells were lysed with Laemmli’s sample buffer (Laemmli, 1970) and polypeptides were separated by 15% SDS–PAGE. The proteins were transferred to nitrocellulose membranes (Towbin et al., 1979). The membrane was blocked with 3% FCS in PBS before it was stained by the method described below.

Peroxidase–anti-peroxidase (PAP) staining method. Sf-9 cells, infected with the recombinant virus or AcNPV, were incubated for 2 days at 28 °C. The cells were fixed with 36% formalin for 10 min and then treated with 0.1% Triton X-100 for 5 min at room temperature (Markoff et al., 1984). Vero cells infected with DEN were incubated for 2 days at 37 °C and then fixed with methanol for 10 min. The cells were stained sequentially with anti-DEN-4 core MAb (Tadano et al., 1989), anti-mouse IgG (rabbit; Dako), anti-rabbit IgG (goat; Cappel), rabbit PAP complex (Jackson Laboratories), and substrate (0.3 mg/ml of 3,3’-dimethylaminobenzidine in PBS plus 0.01% H2O2) as described by Okuno et al. (1978). In some experiments, the anti-DEN-4 core MAb was replaced with antibodies to the purified DEN virion or JE virus or with antibody to the expressed DEN-4 core fusion protein (see below). The optimal dilution of each antibody had been determined previously.

Preparation of the nuclear fraction. Sf-9 cells infected with recombinant virus were incubated for 2 to 3 days. The cells were harvested and the nuclear and cytoplasmic fractions were prepared as described by Borun et al. (1967). A part of the nuclear fraction was stained with Giemsa solution and examined under the microscope to confirm that the preparation was free from cytoplasmic components.

Purification of the DEN-4 core fusion protein and subsequent immunization. The proteins in the nuclear fraction of recombinant virus-infected Sf-9 cells were separated by 15% SDS–PAGE. The DEN core fusion protein which migrated in the 25K region was excised and electrophoretically eluted in a dialysis bag (Maniatis et al., 1982). The eluent was mixed with 0.5 M-KCl to precipitate the SDS–protein complex, which was collected by high speed centrifugation and the pellet was mixed with acetone and repelleted. The pellet was resuspended in a small amount of PBS, transferred to a dialysis bag and dialysed against PBS. The resulting protein solution was mixed with Freund’s complete adjuvant and the mixture was inoculated intraperitoneally (i.p.) into mice. After four additional booster injections with the same preparation, the mice received an i.p. inoculation of Ehrlich’s ascitic tumour cells and the hyperimmune mouse ascitic fluid (HMAF) was harvested 10 days later.

Electron microscopy. Preparation of the samples was as described by Robinson et al. (1987).

RESULTS

Construction of recombinant baculovirus

The DEN-4 cDNA fragment (nucleotide positions 89 to 553) contains the ATG initiation codon of DEN-4 polyprotein at positions 102 to 104. By introducing this fragment into the BamHI site of transfer vector pAc373, the initiation codon of DEN-4 was used for the expression of the DEN-4 core protein (and part of the PreM protein). After cotransfection of Sf-9 cells with mixtures of the transfer vector DNA and AcNPV DNA, the progeny recombinant virus, forming occlusion-negative plaques, was selected. The recombinant virus, designated 373–core, was purified by three successive plaque clonings in Sf-9 cells (Fig. 1).
Expression of dengue virus core protein

Western blot analysis of recombinant virus-infected cells

The proteins of the recombinant virus-infected Sf-9 cells and DEN-4-infected Vero cells were analysed by Western blotting and PAP staining using anti-DEN-4 core MAb. Parental strain AcNPV-infected and mock-infected cells served as controls. A protein band with an $M_r$ of approximately 25K in the recombinant virus-infected cells reacted with the anti-DEN-4 core MAb (Fig. 2, lane 1). Mock-infected and AcNPV-infected cells did not react with this MAb. A protein with an $M_r$ of 15.5K produced in the DEN-4-infected Vero cells also reacted with the anti-core MAb (Fig. 2, lane 4). This protein has been identified as an authentic DEN-4 core protein (Stollar, 1969; Brinton, 1986; Tadano et al., 1989). The difference in $M_r$ between the authentic core protein and the recombinant product was due to the latter protein containing PreM and polyhedrin protein fused to the DEN-4 core protein ($M_r$ 15.5K) at the carboxy terminus.

PAP staining of recombinant virus-infected cells

Sf-9 cells infected with the 373-core recombinant virus were incubated for 24 h at 28 °C and then fixed and stained by the PAP method using anti-DEN-4 core MAb. Mock-infected cells were included as controls. The DEN-4 core fusion protein was present both in the cytoplasm and nucleus of the infected Sf-9 cells (Fig. 3). To confirm this observation, recombinant virus-infected cells were separated into nuclear and cytoplasmic fractions, and each fraction was examined by Western blot analysis using anti-DEN-4 core MAb. The results showed that a large quantity of the DEN-4 core fusion protein migrated to and accumulated in the nucleus (Fig. 4, lane 2).
Purification of the DEN-4 core fusion protein and preparation of antibody

Proteins in the nuclear fraction of the recombinant virus-infected Sf-9 cells were separated by 15% SDS–PAGE. The DEN-4 core fusion protein (Fig. 5, lane 2) was excised and purified and then examined by SDS-PAGE and Western blot analyses. The preparation showed only one protein band which reacted with the anti-DEN-4 core MAb (Fig. 6).

Type-specificity of HMAF and detection of the intranuclear DEN-4 core antigen with HMAF

Vero cells infected with DEN-1, -2, -3, -4 or JE virus were cultured at 37°C until c.p.e. developed in 75% of the cells. The cells were then harvested and examined by Western blot analysis using HMAF specific for the DEN-4 core fusion protein. The HMAF reacted with a protein which migrated to the $M_r$ 15.5K region, identified as the DEN-4 core protein (Fig. 7, lane 4). No reaction was observed with the proteins from DEN-1-, -2-, -3-, JE virus- or mock-infected cells.
Expression of dengue virus core protein

Fig. 4. Western blot analysis of recombinant virus-infected Sf-9 cell lysates probed with the anti-DEN-4 core MAb. Lane 1, whole cell; lane 2, nuclear fraction; lane 3, cytoplasmic fraction. Arrowhead indicates a DEN-4 core fusion protein.

Fig. 5. SDS-PAGE profiles of proteins from Sf-9 cells infected with recombinant virus. Lane 1, whole cell lysate; lane 2, nuclear fraction; lane 3, cytoplasmic fraction; lane M, Mr markers. Arrowhead indicates a DEN-4 core fusion protein.

Fig. 6. SDS-PAGE profile and Western blot analysis of the purified DEN-4 core fusion protein. Lane 1, Coomassie Brilliant Blue-stained gel; lane 2, Western blot probed with DEN-4 core MAb. Lane M, Mr markers.

To examine the intranuclear location of the DEN-4 core protein, DEN-4-infected cells were stained by the PAP method using the core protein-specific HMAF. Clusters of cells in infected cell foci reacted with the HMAF (Fig. 8a). Higher magnification of the cells clearly showed the presence of the intranuclear antigen together with diffuse antigen in the cytoplasm (Fig. 8b). No reaction was observed when DEN-1, -2, -3, or JE virus-infected cells were examined using the
Fig. 7. Western blot analysis of DEN virus-infected and JE virus-infected Vero cell lysates probed with HMAF specific for the DEN-4 core fusion protein. Lane 1, DEN-1; lane 2, DEN-2; lane 3, DEN-3; lane 4, DEN-4; lane 5, JEV; lane 6, mock-infected Vero cell lysates. Arrowhead indicates the DEN-4 core protein.

Fig. 8. PAP staining of Vero cells infected with DEN-3 or DEN-4 virus. The infected Vero cells were cultured as described in Methods. The cells were fixed with methanol and stained. (a) DEN-4-infected cells stained with HMAF specific for DEN-4 core fusion protein; (b) higher magnification of (a); (c) DEN-3-infected cells stained with HMAF specific for purified DEN-3 virions. Bar markers represent 50 μm.
Expression of dengue virus core protein

Fig. 9. Electron micrographs of Sf-9 cells infected with recombinant virus (a) and uninfected cells (b). Bar markers represent 2.5 μm.

HMAF (data not shown). When these same preparations were examined using each homologous polyclonal antibody, the virus-specific cytoplasmic antigens could be observed as illustrated by DEN-3 (Fig. 8c).

Electron microscopy

Electron micrographs of the recombinant virus-infected Sf-9 cells showed no polyhedrin crystalline lattice. The cytoplasm was highly vacuolated, and in the nucleus numerous non-occluded viruses were observed together with the fibrous structures (Fig. 9a).

DISCUSSION

The DEN-4 core protein consists of 112 amino acids (Zhao et al., 1986) of which 24 are basic (e.g. lysine or arginine); the protein is consequently highly basic. The estimated Mr is 13K to 16K (Stollar, 1969; Brinton, 1986). In this experiment, we inserted the DEN-4 core genes and part of the PreM gene into the baculovirus polyhedrin gene region. The recombinant baculovirus directed the synthesis of the DEN-4 core fusion protein with an Mr of about 25K and this retained immunological reactivity with anti-DEN-4 core MAb. The HMAF against the purified DEN-4 core fusion protein reacted with the authentic 15.5K DEN-4 core protein by Western blot analysis. PAP staining of DEN-4-infected Vero cells using the HMAF revealed the presence of the DEN-4 core protein in the nucleus as well as in the cytoplasm. Using a MAb against the DEN-4 core antigen, we observed the accumulation of the DEN-4 core protein in the nucleus (Tadano et al., 1989). The PAP staining pattern of the DEN-4 core protein in the infected cell nucleus, obtained with the HMAF, was similar to that obtained with the MAb. The results indicated that the DEN-4 core protein migrated into the nucleus and localized to form foci in the nucleus. The role of the intranuclear core protein in the course of flavivirus replication is unknown.

The HMAF against the DEN-4 core fusion protein reacted specifically with authentic DEN-4 core protein. The amino acid homology of the DEN core protein among DEN serotypes is 66 to 68% (Mason et al., 1987; Hahn et al., 1988), whereas that between DEN and other flaviviruses is 18 to 44% (Zhao et al., 1986; Mason et al., 1987; Hahn et al., 1988) as deduced from nucleic acid sequence data. Although it has been speculated that the core protein is antigenically similar for the entire genus (Trent, 1977; Russell et al., 1980), the HMAF against the recombinant DEN-4 core fusion protein was found to be type-specific. This can probably be explained as follows.
Only a portion of the epitopes on the authentic DEN-4 core protein may be present in a natural form on the recombinant fusion protein, and confer type specificity. Other DEN-4 core epitopes which are involved in the generic antigenicity may have undergone steric hindrance or conformational changes due to the unnatural structure of the recombinant fusion protein.

A variety of prokaryotic and eukaryotic vectors are now available for the expression of virus genes. One advantage of utilizing the baculovirus expression system is that high levels of expressed product can be obtained (Matsuura et al., 1987). In our case, synthesis of the DEN-4 core fusion protein did not reach these levels. However, the amount of fusion protein expressed in SF-9 cells was significantly greater than that of the authentic DEN-4 core protein observed in lysates of DEN-4-infected cells. We are currently investigating the feasibility of utilizing the DEN-4 core fusion protein as a diagnostic antigen.

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REFERENCES

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