Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies

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Anti-dengue 2 virus core protein monoclonal antibodies (MAbs) reacted with antigens in the cytoplasm and in, or on, the nucleus of dengue 2 and dengue 4, but not dengue 1, dengue 3, Kunjin or Murray Valley encephalitis virus-infected cells. These MAbs also reacted with the core protein from dengue 1, 2 and 4 virions in Western blots. The antigens detected by these MAbs could not be detected in uninfected or heat-shocked cells, but were first detected in infected cells approximately 32 h post-infection. PEPSCAN epitope mapping suggested that all the MAbs react with a region of the dengue 2 virus core protein (RNTPFNMLKRE19) which is adjacent to a putative nuclear localization sequence (KKAR*) and spans a possible second site for the initiation of synthesis of core protein (PFNMLKR*).

Dengue viruses, members of the family Flaviviridae, are a major cause of morbidity and mortality in tropical countries of the world (Halstead, 1988).

Although there is no direct evidence for the involvement of the cell nucleus in flavivirus replication, virus-directed RNA and protein synthesis do occur in the perinuclear region of infected cells (Westaway & Ng, 1980; Ng et al., 1983), and proteins from a number of RNA viruses, including the core protein of dengue 4 virus, have been reported to localize to the nucleus of infected cells (Anzai & Ozaki, 1969; Jackson et al., 1982; Young et al., 1987; Buckley & Gould, 1988; Tadano et al., 1989). However, none of the epitopes recognized by the antibodies used in these studies was defined, raising the possibility of serological cross-reactions between viral epitopes and those of normal cellular proteins (Gould et al., 1983).

Standard protocols (Zola, 1987) were used to derive six IgG1 monoclonal antibodies (MAbs) from hybridomas resulting from the fusion of spleen cells from dengue 2 virus core protein-immunized BALB/c mice and SP2/0-Ag14 myeloma cells.

Dengue 2 virus core protein was obtained by mixing 25 μl sucrose gradient-purified dengue 2 virus (Aaskov et al., 1988) with prestained Mr markers (2 ktl, Bio-Rad) prior to PAGE (Laemmli, 1970), and then, after electrophoresis, eluting protein from a 5 mm × 3 mm strip of acrylamide from immediately in front of the Mr 18 500 prestained Mr marker. The remainder of the gel was stained with Coomassie blue to confirm that the strip of acrylamide recovered contained only core protein.

In a PEPSCAN assay (Geysen et al., 1987), polyclonal sera from mice immunized with dengue 2 virus core protein reacted with many peptides but all six MAbs recognized octapeptides composed of amino acid sequences (Deubel et al., 1986) found in a similar region of the dengue 2 virus core protein (RNTPFNMLKRE19) (Fig. 1). The only protein sequences in the GenBank database to have significant amino acid identity with this region were an insecticidal protein, ISRH-3, from Bacillus thuringiensis (TIFNtwKRE136) (Sen et al., 1988) and a DNA gyrase A subunit from Escherichia coli (TeFNR666) (Swanberg & Wang, 1987). Antibody pairs 6F3.1 and 6F3.2, 4D1 and 2H12, and 4H9.1 and 2E5.1 appeared to recognize similar epitopes, so only one of each pair was used in some subsequent assays.

The MAbs also reacted with the core protein of sucrose gradient-purified dengue 2, dengue 4 and, albeit weakly, dengue 1 viruses (Fig. 2) in Western blots (Aaskov et al., 1988). There was no detectable reaction with dengue 3 virus.

Core protein was first detected by indirect ELISA in acetone-fixed (4 °C, 1 min) cytospin (1000 r.p.m., 2 min) preparations of dengue 2 virus-infected C6/36 Aedes albopictus cells 32 h after infection. MAbs (mouse ascitic fluid diluted 1:20 in PBS pH 7.4) were added to cell monolayers for 45 min followed, after three washes in PBS, by horseradish peroxidase-labelled rabbit antimouse immunoglobulin (Dako). Following a further three washes in PBS, a substrate/chromogen solution of H₂O₂/diaminobenzidine was added to the cell monolayers to localize antibody, and a counterstain of Mayer's
Fig. 1. Reaction of polyclonal mouse serum (a), non-immune mouse serum (b) and MAbs 6F3.1, 6F3.2, 4D1, 2H12, 4H9.1 and 2E5.1 (c to h) with octapeptides composed of overlapping amino acid sequences from the core protein of dengue 2 virus in a PEPSCAN assay. Peptides are numbered from the N terminus of the core protein. The amino acid sequences of the peptides recognized by the MAbs are shown.

Fig. 2. Reaction of MAbs with structural proteins of sucrose gradient-purified dengue 1, 2, 3 and 4 viruses (a to d) in Western blots.

Tadano et al. (1989), although the almost exclusive localization of core proteins to nucleoli was not observed. These results, and the amino acid sequence comparisons discussed above, would seem to exclude the possibility that our MAbs cross-reacted with constituents of host cells such as heat-shock proteins, which are produced in response to virus infection (Garry et al., 1983) or exposure to elevated temperatures (Tissieres et al., 1974) and which may localize to the nucleolus of stressed cells (Milarski & Morimoto, 1989).

Although the determinant 16LKR18 appeared to be critical for the binding of MAbs 6F3.1 and 6F3.2, and 16LKRE19 for the remaining four antibodies, it is possible that adjacent amino acids or protein conformation may also contribute to the function of these epitopes. Predictions of secondary protein structure (Chou & Fasman, 1974; Parker et al., 1986) suggest that the conformation of the dengue 2 virus core protein is more closely related to that of dengue 4 virus than to those of any of the other flaviviruses studied in the region recognized by our MAbs. This may explain the limited cross-reactivity of these MAbs despite dengue 1, 2, 3 and 4, Murray Valley encephalitis and Kunjin viruses sharing the amino acid sequence NMLKR in this region of the core protein (Mason et al., 1987; Osatomi et al., 1988; Zhao et al., 1986; Coia et al., 1988; Dalgarno et al., 1986). Failure of the MAbs to recognize core protein in dengue 1 virus-infected cells when they had reacted with pure virus in Western blots (Fig. 2) may simply reflect the relative amounts of core protein present in each of the assays.

The intracellular distribution of core protein detected with our MAbs contrasts with the generalized cytoplasmic staining observed when using the anti-envelope protein MAb 1B7 (Henchal et al., 1985) (Fig. 3) and
suggests that some of the core proteins remain attached to or inserted in the nuclear membrane rather than being incorporated into mature virions. To examine this possibility, C6/36 cells were disrupted in hypotonic Tris buffer containing deoxycholate and NP40 (Naeve & Trent, 1978) 72 h after infection with dengue 2 virus when nuclear localization of core protein was detectable in $\geq 70\%$ of cells. The resultant nuclei, cytoplasm and a dense membrane aggregate were analysed by Tricine-SDS–PAGE (Schagger & von Jagow, 1987) followed by ELISA on Western blots (Aaskov et al., 1988). A band of core protein of similar $M_r$ was detected in all preparations and in pure dengue 2 virus (Fig. 4).

These data are consistent with the intracellular distribution of staining seen in indirect ELISA with dengue 2 virus-infected cells (Fig. 3) and a flavivirus replication strategy in which core proteins are synthesized in the cytoplasm and then inserted, via their C termini, in the membrane of the endoplasmic reticulum prior to assembly of mature virions and their release from the surface of infected cells (Coia et al., 1988). However, they do not indicate whether the core protein is inside the nucleus or embedded in the nuclear membrane. Previous reports of nuclear localization of flavivirus proteins also failed to demonstrate the presence of these proteins inside the nucleus, although apparent staining of nucleoli is suggestive of internalization (Buckley & Gould, 1988; Tadano et al., 1989).

The dengue 2 virus core protein contains three
putative nuclear localization signals. 8KKAR9 is the same as a signal found in the polyoma virus large T protein (281KKAR285) (Richardson et al., 1986), but would be absent from any core protein truncated at the second methionine residue (Met 15) from the N terminus of the full-length core protein (Castle et al., 1985; Osatomi et al., 1988). The second sequence (73KKSK76) resembles a nuclear localization sequence in nucleo-

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plasm (16KKKK166) (Burglin & De Robertis, 1987), and would be retained even if the core protein was truncated at Met 15 and the 12 or 13 amino acid hydrophilic region at the C terminus was removed prior to incorporation into mature virions (Speight & Westaway, 1989). Dengue 4 virus core protein contains neither of these motifs and yet it localizes to the nucleus of infected cells.

Of potentially greater significance is the bipartite nuclear targeting sequence motif (Dingwall & Lasky, 1991) 85RKkeigrmlnlnRRRR106, variations of which are found in all flavivirus core proteins for which the derived amino acid sequence has been published. However, it remains to be demonstrated experimentally that these putative sequences are required for nuclear localization of dengue virus core proteins. The absence of more reports of nuclear localization of flavivirus core proteins may simply reflect the absence of appropriate reagents to detect these proteins.

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References


Short communication


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