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Immunohistochemical Characterization of a New Monoclonal Antibody Reactive with Dengue Virus-Infected Cells in Frozen Tissue Using Immunoperoxidase Technique

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SUMMARY **This paper presents a novel monoclonal antibody shown to react with cytoplasmic antigens in various dengue infected human frozen organs from autopsy and necropsy specimens. Strong reactivity was found in hematopoietic cells, including immunoblasts, lymphocytes, plasma cells and macrophages of spleen, lymph node, lung, kidney and stomach. Strikingly, strong positivity was demonstrated in cerebral cortex neurones, Purkinje cells, choroid plexus and blood vessels in addition to astrocytes and microglia. Neurotropism of the virus could explain the meningitis, encephalitis, mononeuropathy and polyneuropathy observed by direct toxicity, but noted especially after an activation of mononuclear phagocytes and amplification of the immune response with subsequent vascular inflammation and formation of immune complexes.**

Dengue virus infections are a major cause of morbidity in tropical and subtropical areas of the world. The dengue virus infection causes two forms of illness:dengue fever (DF) and dengue hemorrhagic fever (DHF).[1] Dengue fever is a self-limited febrile disease, while DHF is a severe, sometimes fatal syndrome characterized by hemorrhagic manifestations and plasma leakage that may lead to dengue shock syndrome (DSS). Fluid accumulation in body cavities, thrombocytopenia, and moderate depression of several clotting factors frequently occur in DHF.[2] However, the pathogenesis of dengue virus has not been completely understood and a major impediment is the difficulty in determining the precise cellular target of this virus *in vivo*.

Although it is known that the virus replicates in macrophages *in vitro*, and studies using immunofluorescent antigen detection in frozen tissue have demonstrated that macrophages and macrophage allied cells such as the Kupffer cells, are infected,[3-5] the details of infected cell types in various organs have not been determined. The availability of the monoclonal antibody that identifies these infected cell types in pathological specimens would greatly enhance the immunohistological diagnosis and the pathogenesis study of dengue virus infection.

MATERIALS AND METHODS

Tissues

A complete list of the tissues used in these experiments is shown in Tables 1 and 2. This comprises lymphoid and hematopoietic, lung, kidney, liver and brain tissues, including gastrointestinal organs and all available tissues as listed in Table 3. For the immunohistochemical studies, tissues from 3 autopsies and necropsies of dengue virus infected cases proven by dengue confirmatory

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tests were obtained at Department of Pathology, Maharaj Nakorn Chiang Mai Hospital. The available clinical data are shown in Table 4.

Monoclonal antibody preparation

The monoclonal antibody used in this study was dengue complex-specific (D3-2H2-9-21) obtained from the Hybridoma Cell Bank of the Vector-Borne Diseases Division, CDC, Ft. Collins, Colorado. The dengue virus complex hybridoma-induced ascitic fluid was prepared as previously described.[6] Specificity was determined by indirect immunofluorescent antibody assay at 1:10 dilution using LLC-MK2 cells infected with only dengue viruses. This antibody provided serological confirmation of the dengue virus complex and was unreactive with the other flaviviruses examined.

Immunoperoxidase staining

Frozen sections were stained with the new monoclonal antibody by using horse anti-mouse IgG-IgM avidin-biotin complex, immunoperoxidase-staining reagents (Vector Laboratories, Inc, Burlingame, CA) as described.[7,9] Briefly, the sections were incubated with the primary monoclonal antibody (1:5 dilution) for 60 minutes. After a brief wash in phosphate buffered saline, the slides were treated with the secondary antibody for 30 minutes. The slides were washed and incubated for another 30 minutes with avidin-biotin-peroxidase complex (ABC). After a final wash, the slides were stained with aminoethyl carbazole, 1.2% in acetate buffer containing 0.015% hydrogen peroxide. The slides were then counterstained with Mayer's hematoxylin and cover-slipped with glycerol jelly for examination by light microscopy.

RESULTS

Immunoperoxidase reactivity of the monoclonal antibody on infected human frozen tissues

As shown in Table 1 the strong and predominant staining reactivity of the monoclonal antibody (MAb) in hematopoietic cells were found in the spleen, lymph node, lung, kidney and stomach, but only parenchymal cells of the brain showed strong positivity with MAb. The positive cell type in the spleen were shown to be immunoblasts, lymphocytes and plasma cells in the area of white pulps and macrophages in the red pulps. Similarly, immunoblasts, histiocytes, plasma cells and lymphocytes were

Table 1. Strong reactivity of MAb on infected human frozen tissues.

Organ	Cell types*	
	Parenchymal cells	Hematopoietic cells
Spleen	NR	Immunoblasts Lymphocytes in white pulp Plasma cells Macrophages in red pulp
Lymph node		Immunoblasts, lymphocytes, plasma cells and macrophages
Lung	NR	Alveolar macrophages
Kidney	NR	Immunoblasts, histiocytes, plasma cells and lymphocytes in interstitium and around blood vessels
Stomach	NR	Lymphocytes in mucosa Plasma cells in lamina propria
Brain	- Neurones in cerebrum, - Purkinje's and some granular cells in cerebellum - Astrocytes, microglia - Choroid plexus lining epithelium and vessels	Few lymphocytes in vessels

NR = Negative results

* All defined cell types showed strong positivity +++ as compared to others in Table 2.

Table 2. Mild to moderate reactivity to MAb on infected human frozen tissues

organs	cell types (staining intensity*)	
	Parenchymal cells	Hematopoietic cells
Liver	Liver cells (++)	Kupffer's cells (++)
Small intestine	-	Plasma and mononuclear cells (+) in lamina propria
Large intestine	-	Plasma and mononuclear cells (++) in lamina propria
Appendix	-	Plasma and mononuclear cells (++) in lamina propria

\+ = mild
++ = moderate
\- = negative

Table 3. List of other organs examined with negative reactivity

Organs	Parenchymal cells	Hematopoietic cells
Skin	-	- (Few mononuclear cells in blood vessels)
Breast	-	- (Few mononuclear cells around acini)
Thyroid gland	-	- (Few mononuclear cells in vessels)
Adrenal gland	-	-
Pancreas	-	-
Urinary bladder	-	-
Ovary	-	-
Endometrium	-	-
Uterus	-	- (Few mononuclear cells in vessels)
Heart	-	-
Skeletal muscle	-	-
Smooth muscle	-	-
Diaphragm	-	-

also observed in the follicular and parafollicular areas of the lymph node and interstitium of the kidney, included surrounding blood vessels (Fig.1) Strong positivity of lymphocytes as well as plasma cells were found in the mucosa and lamina propria of the stomach. Only one cell type detected in the lung was proved to be alveolar macrophages. In contrast, the brain was the only organ shown to contain large numbers of dengue virus antigens in parenchymal cells included neurons in the cerebrum (Fig.2), Purkinje's and some granular cells in cerebellum, glial cells, *e.g.* astrocytes and microglia and the last interesting location of antigens in choroid plexus lining epitheluim.

Table 2 shows the mild to moderate staining reactvity of MAb on infected human frozen tissues in certain organs. Plasma cells were among hematopoietic cells found in small and large intestine, including the appendix. Kupffer cells (Fig.3) yielded the moderate staining results and scattered parenchymal cells of the liver also demonstrated moderate staining reactivity with MAb.

There was no localization of dengue virus antigens with this MAb in other organs examined as shown in Table 3. Only a few mononuclear cells in blood vessels were observed in the skin, thyroid gland and uterus. Similarly a few mononuclear cells were also detected around acini of the breast.

Reactivity of MAb with selected human diseases was undertaken. To determine the specificity of MAb

Table 4. Clinical details of three studied patients

Sex	Female	Female	Male
Age (years)	13	5	5
Chief complaint	Fever	Fever	Fever
Onset	1 wk	4-5 days	5 days
Symptoms/Signs	Fever	Fever	Fever
	hematemesis	skin rash	hematemesis
	skin rash	epistaxis	skin rash
Tourniquet's test	Positive	Negative	Positive
Neurological manifestation	Lethargy	Lethargy	Lethargy
Temperature	38 °C	38 °C	38.5 °C
Pulse	120/min	118/min	100/min
Respiratory rate	24/min	34/min	30/min
Blood pressure	100/70	120/70	100/70
Hb/(g %)	15	15.1	14
Hct (%)	46	47	45
WBC (cells/mm^3)	20,000	34,675	45,000
Platelets (cells/mm^3)	80,000	75,000	104,000
Treatment	Fluid	Fluid	Fluid
	Steroid	Steroid	Steroid

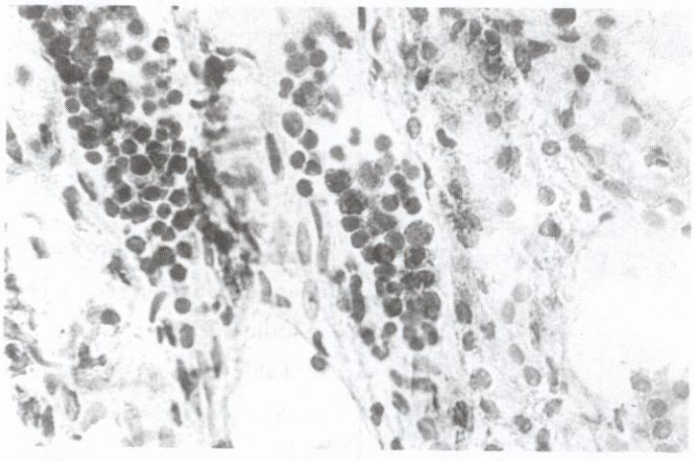

Fig. 1 Immunoperoxidase staining reactivity of dengue monoclonal antibody on frozen tissue sections. All were counterstained with hematoxylin.Kidney: immunoblasts, macrophages and mononuclear cells were demonstrated in the interstitium and around blood vessels (x400).

against dengue antigens, this reagent was used to stain a variety of human diseases using fresh tissues from autopsy specimens. Those cases are disseminated herpes simplex virus, disseminated aspergillosis, hepatocellular carcinoma with generalized metastasis, breast carcinoma with metastasis and disseminated tuberculosis. Only one case of each disease was available for the study. MAb did not show any positivity in these cases.

DISCUSSION

Previous monoclonal antibodies have been used to dissect the antigenic relatedness between flaviviruses and define epitopes on viral proteins

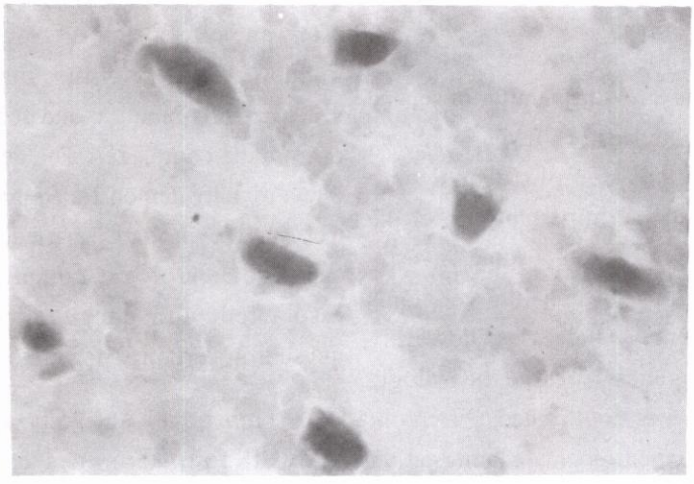

Fig. 2 Immunoperoxidase staining reactivity of dengue monoclonal antibody on frozen tissue sections. All were counterstained with hematoxylin. Cerebral cortex neurones showed strong positive staining with MAb (x400).

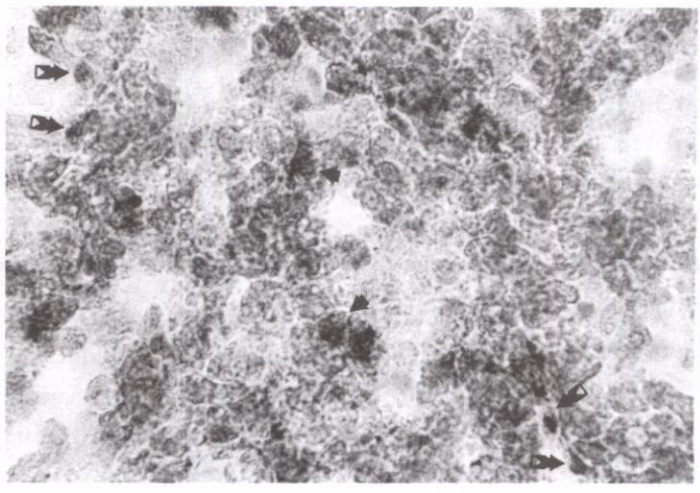

Fig.3. Immunoperoxidase staining reactivity of dengue monoclonal antibody on frozen tissue section. All were counterstained with hematoxylin. Liver: scattered positivity in **Kupffer's** cells and liver cells (x400).

involved in a range of biological activities. Various techniques have been applied to the identification of the primary sequences involved in monoclonal antibody binding but none to the distribution of antigens on various human infected organs. Previous studies of tissue localization using immunofluorescent technique only detected dengue virus antigen positive monocyte-like cells associated with glomerular basement membranes and in mononuclear cells closely infiltrated around blood vessel walls in dermal papillae.[10,11] Dengue virus was also demonstrated by immunflorescence in Kupffer cells, sinusoidal lining cells of the spleen and alveolar macrophage of the lung.[12,13]

We have shown in this study that dengue viruses can infect human hematopoietic cells including immunoblasts, lymphocytes, plasma cells and macrophages in various organs. Some transformed lymphocytes and immunoblastic proliferation are B cell in orgin (CD 20+) while focal areas of mature T cell lymphocytic infiltrate are also observed. Suvattee[14] reported that buffy coat preparations of dengue-infected patients contained approximately 20% blast cells showing the evidence of B-cell lineage by the presence of immunoglobulin rings without T-rosette forming. Other investigators also found that dengue viruses replicated in B cell lineage and Raji (B lymphoblastoid) cells.[15,16] Those hematopoietic cells containing dengue viral antigens are mainly found in the spleen, lymph node, lung, kidney and stomach. Dengue viral antigens were also detected along

the gastrointestinal tract including the appendix and small and large intestines.

Bhamarapravati and others,[17,18] in a study of 100 autopsies of patients who died of DHF/DSS, reported that the gastrointestinal tract was the region most frequently involved (in 65% of the cases). Beside the mononuclear phagocytic cells in the spleen, lymph node, liver, lung, kidney and gastrointestinal tract where dengue viral antigens can be localized, the viruses do not show other organotropisms with the possible exception of the liver and the brain. In the liver cells, moderate staining reactivity was detected focally, supported by the presence of dengue virus RNA in the previous study.[19,20] Strikingly the specific viral neurotropism demonstrated in this study included the cerebral cortex neurones, Purkinje's and some granular cells in the cerebellum together with astrocytes and microglia. We also found strong positive viral antigens in choroid plexus which is the well recognized area for immune complex deposition.[21,22]

Besides the epithelial lining of the choroid plexus, viral antigens were found in endothelial cells of blood vessels along the plexus. To our knowledge this is the first report showing dengue viral antigens targeted to endothelial cells as it has been shown for influenza virus.[22] Electron microscopy has shown that endothelial cells in skin biopsies from DHF patients have increased number of vacuoles and pinocytotic vesicles; these are important in the transport of plasma fluids from the capillary to the pericapillary space.[24]

Certain neurologic signs and symptoms are classically observed during acute dengue fever infection.[25] Neurological complications were shown to be an important part of the clinical spectrum and some investigators reported encephalitic symptoms in 70% of their fatal cases; however, the pathogenesis of the neurologic disorders was not defined.[26] From our study those neurological manifestation could be explained by viral neurotropism and immunologic reactions. Several studies have shown that at appropriate levels of dengue viral antigen, the subsequently formed immune complexes show a correlation with disease severity.[27] A reduction in the circulating complement factors C3, C4 and C5 has also been shown to be correlated with disease severity.[2,28,29] The attachment of immune complexes or complement components to endothelial cells and platelets could induce the subsequent destruction of these blood components and thus contribute to the pathologic manifestations. Following activation of mononuclear phagocytes and amplification of the immune response to the virus, the production of soluble leukocytic factors such as thromboplastin, permeability factor, and histamine might account for the major physiopathologic findings in DHF/DSS. However, some investigators attribute the DHF/DSS to the virulence of dengue virus rather than immunopathology. Neurotropism of the virus could also explain the meningitis, encephalitis, mononeuropathy[29] and polyneuropathy observed by direct toxicity, but noted especially after an appropriately responsive immune reaction with subsequent vascular inflammation and formation of immune complexes. The degree of direct toxicity demonstrated by neurotropism are probably varied among cases so neurologic complications associated with dengue fever are unusual in certain reports.[25,30,32]

This novel antibody specific with all four serotypes could be fruitful in immunodiagnosis of dengue infected human tissues and providing us a better insight in pathogenesis of the DHF/DSS. Dengue fever and its complications could probably be eliminated with the development of a specific vaccine containing all four serotypes of dengue viruses. Further attempt is to make this monoclonal antibody retain their reactivity in paraffin embedded sections. The availability of monoclonal antibody to tissue dengue virus antigens would help in relating the clinical studies of a number of investigators.

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