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IMMUNOFLUORESCENCE STUDIES ON THE REPLICATION OF SOME ARBOVIRUSES IN NUCLEATED AND ENUCLEATED CELLS

V. J. LAD, A. K. GUPTA*, S. N. GHOSH, K. BANERJEE

National Institute of Virology, 20-A, Dr. Ambedkar Road, P. B. No. 11, Pune-411 001, India

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Summary. – Porcine stable kidney (PS) or Vero cells infected with either flavi-(Japanese encephalitis – JE, West Nile – WN, and Dengue – DEN-2) or alphaviruses (Chikungunya – CHIK and Sindbis – SIN) were stained in indirect fluorescent antibody (FA) assay with anti-JE virus monoclonal (MoAb) Hx-3 (flavivirus cross-reactive) and polyclonal (immune PF) antibodies. By 48 hr post infection (p.i.), 15 to 20 % of the three flaviviruses and CHIK virus infected cells, which revealed positive cytoplasmic immunofluorescence (IF), showed intranuclear IF. By 24 hr p.i., the intranuclear IF was not observed or became diminished. The enucleation of cells by cytochalasin B treatment prior to the infection with any of the three flaviviruses resulted in the loss of IF compared with the cells enucleated after the infection (18 hr p.i.) whereas SIN or CHIK virus-infected cells reacted similarly by the either method. These findings indicate an essential role of the nucleus in the replication of the flaviviruses only and while replicating in the infected cells, flaviviruses and CHIK virus might express viral specific proteins in the cell nuclei.

Key words: arboviruses; nuclear immunofluorescence; enucleated cells

Introduction

The arboviruses belonging to two genera – alphaviruses and flaviviruses (family Togaviridae), represent a major public health problem in many countries including India (Padbidri *et al.*, 1973; Moath, 1986). The replication patterns of flavi- and alphaviruses in cells reveal that the flaviviruses have a considerably longer cycle compared to that of alphaviruses (Pfefferkorn, 1968). In addition, electron microscopic (EM) studies carried out by Yasuzumi and Tsubo (1965*a, b*) have shown the presence of viral precursors in the nuclei of JE-virus infected cells during the latent phase but none of these and other studies clearly reveal the role of nucleus in the replication of both flavi- and alphaviruses

* For correspondence and reprint requests

(Takeda *et al.*, 1965; Murphy *et al.*, 1968; Matsumura *et al.*, 1971; Nishimura and Tsukeda, 1971). Employing anti-JE virus MoAb and immune PF, Gupta *et al.* (1991) demonstrated nuclear IF in 15 to 20 % of JE virus-infected cells positive for the cytoplasmic IF, thus suggesting the role of nucleus in replication of the virus. In the present study, we report the nuclear IF in the cells infected with flavi- (JE, WN and DEN-2) and alphaviruses (CHIK but not SIN). The enucleation of cells by the cytochalasin B treatment, prior to the infection with any of the three flaviviruses results in the loss of IF compared with the cells enucleated 18 hr after the infection, whereas SIN or CHIK virus-infected cells reacted similarly by the either method.

Materials and Methods

PS and Vero cells were maintained in Earle's based minimum essential medium (MEM) containing 10 % goat serum. The cells were used at their 10th to 15th passage for preparing cell monolayers and were infected with the viruses as described earlier (Gupta *et al.*, 1991). For JE (733913 strain), WN (68856) and SIN (AR 339) viruses, PS cells were employed, whereas for DEN-2 (P23085) and CHIK (634029) viruses, Vero cells were used. The uninfected and infected cells were stained by indirect fluorescent antibody (FA) technique with immune PF raised against all these viruses (Gupta *et al.*, 1991). Immune PF were obtained from the ascites produced after injecting Ehrlich's ascitic tumour cells to the immunized mice. Anti-JE virus MoAb, Hx-3 (H-haemagglutination-inhibiting, X-flavivirus cross-reactive) (Kedarnath *et al.*, 1986) was also included. The culture supernatant collected from SP2/0 cells and normal PF of unimmunized mice were included as negative controls.

The enucleated cells were obtained after 18 hr p.i. (A) by the treatment with cytochalasin B according to our modification of the procedure of Kos *et al.* (1975). The cell monolayers on round coverslips (14 mm in diameter) were infected with the viruses. At 18 hr p.i., a coverslip was inserted into a plastic tube containing Dulbecco's MEM with 10 % foetal calf serum (FCS) and 15–20 μg/ml cytochalasin B (Sigma). The cell monolayer was oriented towards the bottom of the tube to ensure the contact of cells with medium. The cells were immediately centrifuged at room temperature at 5000 rpm for 1 hr. Subsequently, the coverslips were washed in MEM and placed at 37 °C in Petri dishes containing Dulbecco's MEM with 20 % FCS. The enucleated cells after fixation in chilled acetone were stained for indirect FA with immune PF and anti-JE virus MoAb Hx-3 as mentioned earlier. An autoreactive anti-JE virus MoAb NHA-1 (NH-non HI, A-autoreactive) (Kedarnath *et al.*, 1986) which reacts with almost 95 % of the cell nuclei, was included to check the degree of enucleation obtained by the cytochalasin B treatment of cells.

The uninfected cells were also enucleated employing 10 μg/ml cytochalasin B and subsequently infected with the viruses. The enucleated cells were stained for indirect FA after 18 hr p.i. (B) with the viruses and were compared for the IF with the cells enucleated after the infection (A).

Results

None of the uninfected control cells showed IF when stained with immune PF or with MoAb Hx-3. The cells infected with either of the flavi- or alphaviruses showed cytoplasmic IF with group specific immune PF 24 hr p.i. (Table 1). The cytoplasmic IF was further intensified along with the increase of IF-positive cells 48 hr p.i. for any of the three flaviviruses. By 72 hr p.i., the most of IF material was located extracellulary probably due to the advanced cytopathic effect (CPE)

Table 1. Immunofluorescence in nucleated cells

Cells infected with viruses	p.i. (hr)	Immune PF raised against viruses										Anti-JE virus MoAb Hx-3	
		JE		WN		DEN-2		SIN		CHIK			
		Cyto	N	Cyto	N	Cyto	N	Cyto	N	Cyto	N	Cyto	N
1. JE*	24	3+	2+	2+	1+	2+	1+	ND	ND	ND	ND	2+	2+
	48	4+	1+	3+	1+	2+	1+					3+	1+
2. WN	24	2+	1+	2+	2+	ND	ND	ND	ND	ND	ND	1+	W
	48	3+	-	3+	1+							1+	W
3. DEN-2	24	2+	1+	ND	ND	2+	1+	ND	ND	ND	ND	1+	W
	48	2+	-			2+	-					1+	W
4. SIN	24	1+	-	ND	ND	ND	ND	2+	-	ND	ND	W	-
	48	ND	ND					ND	ND			ND	ND
5. CHIK	24	ND	ND	ND	ND	ND	ND	ND	ND	3+**	1+**	-	-
	48									ND	ND	ND	ND

Cyto = cytoplasmic; N = nuclear; ND = not done; W = weak; - = negative
Infected PS cells showed no IF with normal PF and SP2/O cell culture supernatant.
* PS cells infected with JE virus were used as a positive control for both cytoplasmic and nuclear IF.
** Similar results were obtained also with anti-CHIK MoAb.

of viruses which started 48 hr p.i. In the case of alphaviruses by 48 hr p.i., the most of cells was degenerated. Interestingly, 15–20 % of the three flaviviruses and CHIK virus infected cells which revealed positive cytoplasmic IF, simultaneously showed intranuclear IF by 24 hr p.i. By 48 hr p.i., the intranuclear IF was diminished or got obliterated. Anti-JE virus MoAb Hx-3 reacted similarly with all the three flaviviruses. Interestingly, the MoAb Hx-3 also reacted, though weakly (cytoplasmic IF) with the cells infected with SIN virus. The positive nuclear IF in CHIK virus-infected cells stained with the immune PF was confirmed by staining the cells with anti-CHIK MoAb for indirect FA.

The cells enucleated 18 hr p.i. (A), infected with any of the flavi- and alphaviruses showed cytoplasmic IF with the group-specific immune PF. The MoAb Hx-3 also reacted similarly with the enucleated cells infected with any of the three flaviviruses (Table 2). The cells infected with the viruses after enucleation with cytochalasin B (B), showed cytoplasmic IF only for the alphaviruses and none for the three flaviviruses. The cells enucleated after infection with viruses (A) required higher concentrations (15 to 20 μg/ml) of cytochalasin B compared to those enucleated before (B) the infection (10 μg/ ml). Also the enucleated cells were considerably smaller then the nucleated cells. The autoreactive anti-JE virus MoAb NHA-1 showed nuclear IF

Table 2. Cytoplasmic immunofluorescence in enucleated cells

Cells infected with viruses	Enucleated before (B) or after (A) infection	Immune PF raised against virus					Anti-JE virus MoAb Hx-3
		JE	WN	DEN-2	SIN	CHIK	
1. JE	B	-	-	-	ND	ND	-
	A	2+	1+	1+			1+
2. WN	B	-	-	ND	ND	ND	-
	A	1+	1+				W
3 DEN-2	B	-	ND	-	ND	ND	-
	A	1+		2+			W
4. SIN	B	1+	ND	ND	1+	ND	W
	A	W	ND	ND	1+	ND	W
5. CHIK	B	ND	ND	ND	ND	2+	-
	A					2+	-

- = negative, W = weak; ND = not done

only with 5 % of the cytochalasin B treated cells as compared to 95 % of the nucleated cells thus indicating that the enucleation of cells was almost complete.

Discussion

Suri and Banerjee (1987) have carried out the IF studies on JE, WN or DEN-2 virus infected mouse embryo brain cells, employing immune PF. They recorded both the nuclear and cytoplasmic IF in the cells infected with JE and DEN-2 viruses whereas for WN virus, only cytoplasmic IF was observed. In the present study, the demonstration of intra-nuclear IF in the three flaviviruses (JE, WN and DEN-2) infected cells by 24 hr p.i. emphasizes an essential role of nucleus in the replication of these viruses. Interestingly, the flavivirus cross-reactive MoAb Hx-3 showed cytoplasmic IF with SIN virus infected cells, indicating that the MoAb recognizes a cross-reactive epitope present in some of the alphaviruses.

The experiments carried out with enucleated cells also indicate that both the alpha- and flaviviruses replicated and produced viral antigens (IF positive) in the cells enucleated after the infection. However, the cells enucleated before the infection supported the replication of alphaviruses only was observed by the presence of cytoplasmic IF. These findings indicate an essential role of the nucleus required for the replication of the flaviviruses only. Also the flaviviruses and CHIK virus while replicating in the infected cells might express some viral

specific proteins in the cell nuclei, probably suggesting an essential role of nucleus in transcriptional and post-transcriptional changes of mRNA formed by the viruses. Studies carried out on cells infected with Zika or Langat (Buckley and Gould, 1988), JE (Gould *et al.*, 1983) and DEN-4 viruses (Tadano *et al.*, 1989) indicate that such virus specific proteins are also expressed in nuclei of infected cells. The detailed studies on these aspects are thus to be carried out to understand better the role of such mechanisms in the pathogenesis of arbovirus diseases.

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