Acta virol. **36:** 401-411, 1992

MONOCLONAL ANTIBODY TO JAPANESE ENCEPHALITIS VIRUS CROSS-REACTING WITH HISTONES PRESENT IN THE CELL NUCLEI

A. K. GUPTA, S. BHATTACHARYA, V. J. LAD, S. A. SARTHI, M. KUTUBUDDIN, S. N. GHOSH, K. BANERJEE

National Institute of Virology, 20-A, Dr. Ambedkar Road, P. B. No. 11, Pune-411001, India

Received September 26, 1991

Summary. - An immunoglobulin G (IgG2b) class of monoclonal antibody (MoAb, NHA-1) raised against Japanese encephalitis virus (JEV) E glycoprotein, reacted with the viral antigen expressed in cytoplasm of the infected cells and also with the cell nuclei, by an indirect fluorescent antibody technique (FA). The NHA-1 reactivity to nuclei was found to be due to its recognizing a JEV cross-reactive epitope present on the nuclear histones. Adsorption with calf thymus histones (type II-AS) showed a drop in NHA-1 reactivity to both JEV and histones by an enzyme-linked immunosorbent assay (ELISA) and indirect FA; the drop was higher against the histones. The MoAb recognized specifically the viral antigens expressed on the infected porcine kidney cell surface by a modified indirect FA. ELISA carried out with glutaraldehyde-fixed antigens showed an almost 2-fold increase in the reactivity over unfixed JEV antigen but none for the histones. Thus, the results indicate that histones share a sequential homology with E glycoprotein of JEV, which might lead to an autoimmune disorder induced due to the molecular mimicry between these two antigens.

Key words: monoclonal antibodies; Japanese encephalitis virus; histones; molecular mimicry

Introduction

Antigenic similarity or "molecular mimicry" between the antigens of infectious agents and host tissues has been invoked to explain the induction of autoimmune diseases by the microbes (Tan, 1982; Shoenfeld *et al.*, 1986; Oldstone, 1987). Such relationships have been demonstrated by the detection of cross-reactive epitopes using MoAbs.

Many viral proteins share epitopes with host-cell proteins. In an analysis of over 600 MoAbs raised against a number of viruses, Srinivasappa *et al.* (1986) found that nearly 4 % of the MoAbs cross-reacted with host determinants

expressed in uninfected mouse tissues. Thirty four MoAbs raised against JEV were also tested against mouse tissues and six of these showed positive reaction. Gould *et al.* (1983) reported an IgM type of MoAb prepared against non-structural (NS-1) protein of JEV, which recognized the viral antigen expressed inside cytoplasm of infected cells as well as an autoreactive epitope present in the nuclei of infected/uninfected cells. We report an IgG2b type of MoAb raised against structural protein (E glycoprotein) of JEV which reacts with the viral antigen expressed in cytoplasm of the infected cells and also recognizes a cross-reactive epitope on the histones present in the cell nuclei.

Materials and Methods

Cells and cell cultures. Vero and porcine stable kidney (PS) cells maintained at the National Institute of Virology, Pune, were grown in Earle's based minimum essential medium (MEM) containing 10 % goat serum. The mosquito (*Culex bitaeniorhynchus*) cells grown in Mitsuhashi and Maramorosch (MM) medium containing 10 % foetal calf serum (FCS) (Pant and Dhanda, 1984) were also employed during the studies. Primary chicken embryo fibroblasts (CEC) were prepared by trypsinization of 10 day old embryos. The vertebrate cells were incubated at 37 °C whereas mosquito cells at 28 °C.

Viruses. Two Indian strains of JEV, P20778 (Webb *et al.*, 1964) and 733913 (Banerjee *et al.*, 1976), isolated from the human brains of JE cases were employed in the experiments. Virus pools were prepared and assayed in PS cells by recording cytopahtic effect and stored at -70 °C. For ELISA, JEV (P20778) grown in PS cells and purified by the sucrose density gradient centrifugation, was used as antigen.

Anti-JEV MoAb. Tissue culture fluids (TCF) were collected from anti-JEV hybrid clones (IIIG9H4B12 or IIIG9H4A2) (Kedarnath *et al.*, 1986; Cecilia *et al.*, 1988) maintained in Dulbecco's MEM containing 10 % FCS. Ascitic fluid (AF) was also obtained from the ascites produced in pristane-primed BALB/c mice after the inoculation of cloned cells. The reactivity of MoAb, designated as NHA-1 (NH - non-haemagglutination-inhibiting, A - autoreactive), to JEV antigen was initially screened by ELISA. The MoAb was found to be of IgG2b subclass in agar gel diffusion test performed against monospecific anti-mouse immunoglobulin sera (Dakopats).

ELISA. Both TCF and AF of MoAb NHA-1 were tested in ELISA for the antibodies to JEV as described earlier (Kedarnath *et al.*, 1986), employing 1 μg/well of the antigen for coating the wells. Uninfected PS cell culture supernatant served as a negative control. The MoAb was also tested for the reactivity against a number of compounds (Table 1) similarly, except that the wells were coated with one of them (1 μg/well). NHA-1, in addition to JEV, was found to react with histones (calf thymus type II-AS, Sigma) and not with others.

Indirect FA. JEV (733913) infected and uninfected cell monolayers were subjected to the standard indirect FA with MoAb NHA-1 as described earlier (Gupta *et al.*, 1991*a*). TCF of Sp2/0 cells was included as a negative control. The enucleated cells obtained by cytochalasin B (Sigma) treatment (Kos *et al.*, 1975) were tested similarly for the immunofluorescence (IF). Also, the metaphase chromosomal preparations obtained by colchicine treatment from Sp2/0 cells were subjected to indirect FA against NHA-1.

Histone depletion by acid treatment and subsequent reconstitution with the histones were carried out according to the method described by Tan *et al.* (1976), modified so as to obtain optimum results. Briefly, the acetone-fixed PS cell monolayers were treated with 0.2 N HCl for 1 hr under continuous stirring on a magnetic stirrer. For histone reconstitution studies, the cells after washing thoroughly with 0.15 mol/l phosphate buffered saline (PBS pH 7.2), were allowed to react with histone (type II-AS) solution (10 μg/ml in PBS) at room temperature for 1 hr. Histone-depleted and the reconstituted cells were compared for the IF against NHA-1.

NHA-1 adsorption with histones. AF of MoAb NHA-1 diluted 1:25, was mixed with an equal volume of histones type II-AS (5 μg/ml) and the mixure was kept overnight at 4 °C. The adsorbed MoAb was tested both by indirect FA and ELISA for the reactivity against JEV antigen and histones. The unadsorbed diluted MoAb (1:50 in PBS) served as a control. Also higher dilutions (1/500, 1/1000 and 1/10 000) of the adsorbed and unadsorbed MoAb were tested similarly.

Modified indirect FA. JEV infected and uninfected cell monolayers were subjected to the modified indirect FA with MoAb NHA-1 for the demonstration of viral antigens on the surface of infected cells as described earlier (Gupta *et al.*, 1991*b*). Briefly, the infected cells were treated with the MoAb in presence of sodium azide and after thorough washing, the cells were fixed in chilled acetone. The cells were further treated with FITC-conjugated anti-mouse immunoglobulins similarly as in the case of standard indirect FA.

ELISA with glutaraldehyde-fixed antigens. To prevent the conformational changes in the epitopes present on unfixed antigens, an ELISA was carried out with MoAb NHA-1 employing glutaraldehyde-fixed histones (type II-As) and JEV antigen according to the method described by Cepica *et al.* (1990). The wells were coated with 1 μg/well of the antigen and before blocking, these were fixed in 1 % glutaraldehyde in PBS at room temperature for 1 hr. The antigen fixed with (5 %) methanol served as a conrol. The results were expressed as percentage of the unfixed antigen.

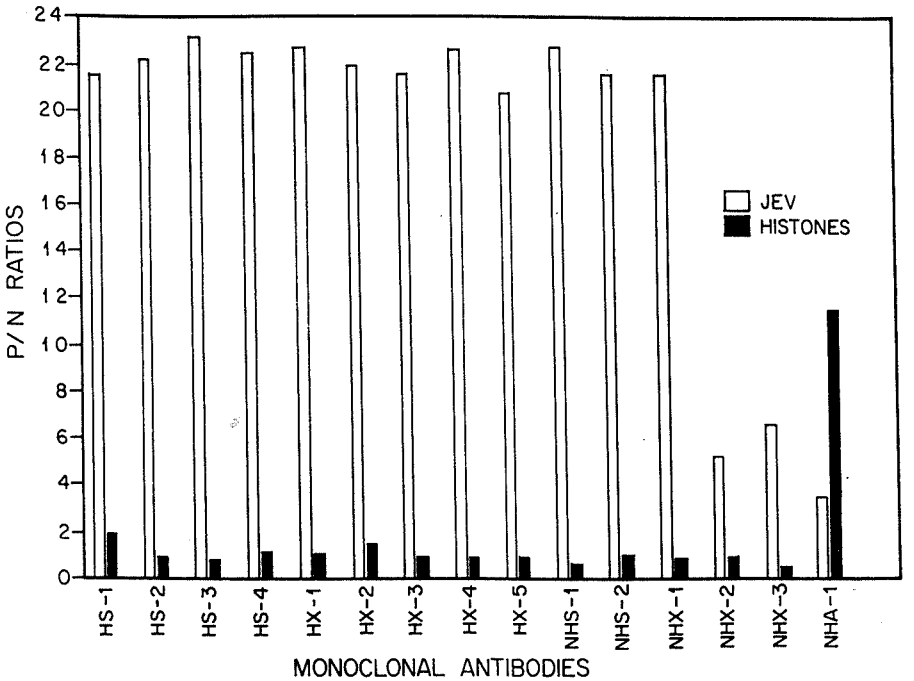

Fig. 1
Reactivity of anti-JEV MoAbs (TCF)

Results

MoAb NHA-1 reactivity in ELISA

The TCF of MoAb NHA-1 reacted both with histones (type II-AS) and JEV antigen but not with DNA, RNA and other proteins (Table 1) indicating the bi-specific activity of the MoAb. The MoAb showed higher reactivity against the histones than with JEV (Fig. 1). The TCF of other MoAbs specific to JEV, namely Hs-1 to 4 (H - haemagglutination-inhibiting, s - specific), NHs-1 and 2 or flavivirus cross-reactive MoAbs Hx-1 to 5 and NHx-1 to 3 (x - flavivirus cross-reactive) reacted only with JEV antigen and none with the histones. However, AF of these MoAbs and even the normal mouse serum reacted occasionally with histones, although to a lesser extent compared to the AF of NHA-1 (data not shown).

MoAb NHA-1 (1:50 diluted), after adsorption with histones showed decrease in reactivity to both the histones (64.3 %) and JEV (52.1 %) (Fig. 2). Such a drop in the reactivity to histones was greater than that against JEV antigen. However, with higher dilutions (1:500 and 1:1000) of the adsorbed MoAb, the decrease in the reactivity was equal or higher against JEV.

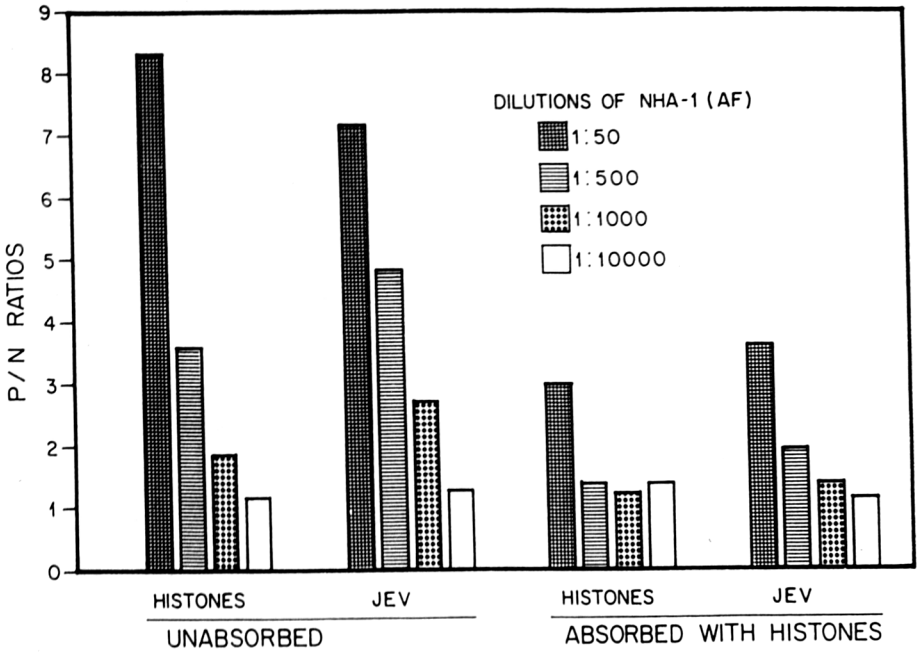

Fig. 2
Decrease in NHA-1 reactivity after adsorption with histones

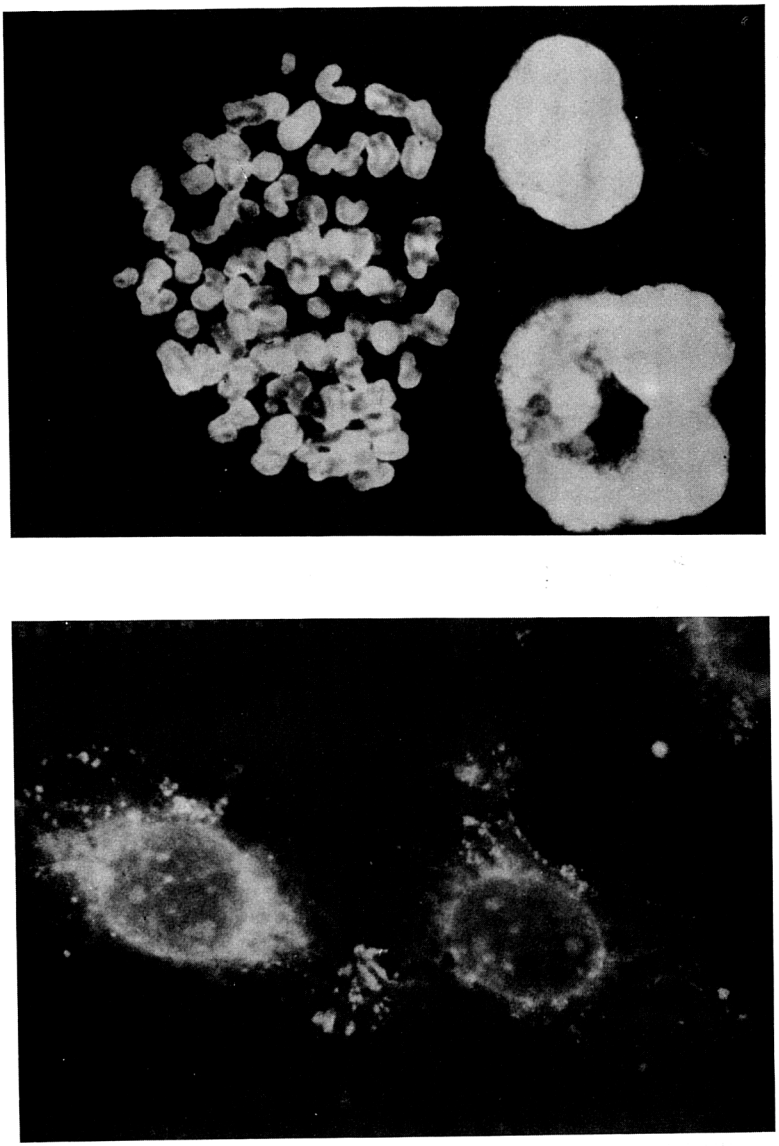

Fig. 3
Immunofluorescence with NHA-1 (× 1000)
Fig. 3-1. Sp2/0 cells, fixed metaphase chromosomal preparation.
Fig. 3-2. JEV-infected PS cells, cytoplasmic IF to JEV antigen and nuclear IF to histones.

Table 1. Anti-JEV MoAb NHA-1 (TCF) reactivity to different compounds tested in ELISA

No.	Antigens	A		P/N*
		NHA-1	Sp2/O	
1	Calf thymus DNA	0.19	0.14	1.36
1	Bacterial DNA	0.28	0.35	0.80
3	Histones (type II-AS)	1.13	0.15	**7.53**
4	Human albumin	0.17	0.22	0.77
5	Human gamma-globulin	0.19	0.19	1.00
6	Myoglobin	0.19	0.15	1.27
7	Ovalbumin	0.25	0.22	1.14
8	RNA	0.15	0.14	1.07
9	Purified JEV antigen	0.40	0.15	**2.67**
10	Uninfected PS culture supernatant	0.25	0.16	1.56

A - absorbancy
* A of the MoAb/A of Sp2/0

IF of uninfected and infected cells

Uninfected Vero, PS, CEC and mosquito cells showed nuclear IF with NHA-1 which was found to be confined to the chromosomes (Fig. 3-1). With PS infected cells, the MoAb showed both the nuclear and cytoplasmic IF (Fig. 3-2); the cytoplasmic IF, however, was masked by the former. Also the MoAb showed the cytoplasmic IF with PS cells enucleated after JEV infection. Other MoAbs (Hs, Hx, NHs and NHx) showed only cytoplasmic IF with the infected PS cells and none with uninfected cells (Gupta *et al.*, 1991*a*). The AFs of these MoAbs which were occasionally positive for the anti-histone activity in ELISA, were negative for such activity by the indirect FA.

Histone depletion of uninfected PS cells by HCl treatment showed loss of nuclear IF (Fig. 4-2) which was restored when acid-treated cells were reconstituted with histones, prior to the treatment with NHA-1 (Fig. 4-4). The lysine-rich histone was found to be more efficient as compared with the arginine-rich histone, in restoring the IF of histone-depleted cells. Also the adsorption of NHA-1 with histones, showed the loss of nuclear IF against uninfected PS cells (Fig. 4-3).

Detection of viral antigens on cell surface

NHA-1 showed IF on the surface of cells infected with JEV (733913) (Fig. 5) which was similar to that observed with MoAbs Hs-1 and Hx-3 and polyclonal antibodies. Uninfected cells did not react either with NHA-1 or with other anti-JEV MoAbs and polyclonal antibodies. NHA-1 did not react with the nuclei of the uninfected/infected cells due to its inability to penetrate inside the unfixed cells (Hudson and Hay, 1976).

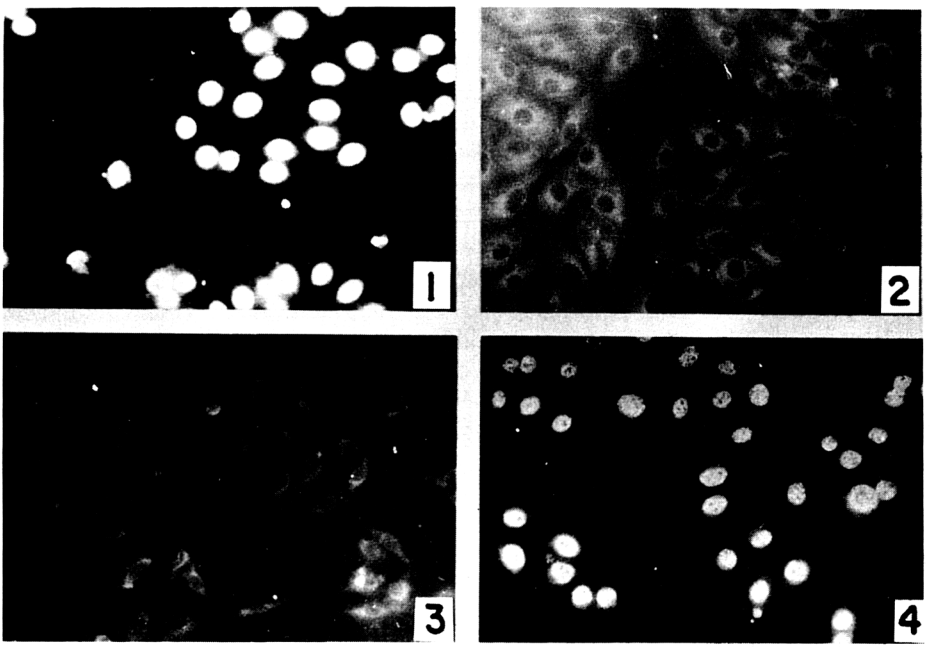

Fig. 4
Nuclear immunofluorescence of uninfected PS cells with NHA-1 (× 100)
Fig. 4-1. Control (treatment with NHA-1 only).
Fig. 4-2. Cells pretreated with acid.
Fig. 4-3. NHA-1 adsorbed with histones.
Fig. 4-4. Cells pretreated with acid and reconstituted with histones

Fig. 5
NHA-1 showing surface immunofluorescence with JEV-infected PS cells by the modified method (× 400)

Table 2. Increase in reactivity to glutaraldehyde-fixed JEV antigen in ELISA

MoAb (TCF)	Purified JEV antigen			Histones		
	Unfixed*	Fixed*	Percentage of unfixed**	Unfixed*	Fixed*	Percentage of unfixed**
Hs-1	8.6	2.9	33.7	1.4	1.3	92.9
-2	8.5	2.8	32.9	1.3	1.3	100
-3	7.0	3.1	54.3	1.3	1.3	100
-4	5.7	2.3	40.4	1.1	1.2	109.1
Hx-1	3.9	1.9	48.7	1.1	1.1	100
-2	3.3	1.7	51.5	1.2	1.1	91.7
-3	3.3	1.4	52.4	1.0	1.0	100
-5	3.6	1.6	44.4	1.0	1.1	110
NHx-1	3.4	1.3	38.2	1.4	1.3	92.9
NHs-1	3.5	2.1	60.0	1.2	1.2	100
-2	3.2	2.1	65.6	1.1	1.2	109.2
NHA-1	**1.6**	**3.1**	**193.7**	2.4	2.5	104.2

TCF - tissue culture fluid supernatants of anti-JEV clones
* P/N ratio
** 25 % of more increase or decrease was taken as significant based on ELISA carried out on methanol-fixed JEV antigen and histones (increase or decrease ranged between 5.3 to 11.2 %)

Increase in reactivity against fixed JEV antigen

The ELISA results with glutaraldehyde-fixed JEV antigen showed an almost two fold increase in NHA-1 reactivity compared to the unfixed antigen (193.7 %). The MoAbs belonging to other group (Hs, Hx, NHs and NHx) showed a decrease in the reactivity ranging from 34.4 % to as high as 67.1 % (32.9 % to 65.6 % reactivity of the unfixed JEV antigen) (Table 2). In contrast, the reactivity of NHA-1 and other MoAbs to glutaraldehyde-fixed histones did not show significant change (≥ 25 % difference). The methanol-fixed antigens included as controls, showed almost similar values as observed with the unfixed antigens (increase or decrease ranged from 5.3 % to 11.2 %).

Discussion

In the present study, anti-JEV MoAb NHA-1 (IgG2b subclass) raised against E glycoprotein of JEV, cross-reacted with the histones present in the cell nuclei. The adsorption of the MoAb with the histones (type II-AS) showed a drop in reactivity to the histones which was higher than that to JEV antigen, thus indicating a higher affinity of the MoAb towards the former. The MoAb recognized specifically the JEV antigens on the surface of infected cells by the modified IF test (Gupta, *et al.* 1991*b*). Also, the experiments with ELISA

employing glutaraldehyde-fixed antigens showed an almost 2-fold increase in NHA-1 reactivity with JEV antigen and none with histones, thus confirming the conformational changes in the epitope being responsible for the low or no reactivity by indirect FA and radio-immunoprecipitation assay, respectively. The increase in NHA-1 reactivity to glutaraldehyde-fixed antigen seems to be due to the cross-linking in the epitope (Cepica *et al.*, 1990). Studies carried out recently by Ghanekar *et al.* (1991) on affinity-purified polyclonal anti-idiotypic (Ab2) antibodies raised in rabbits against MoAb NHA-1 (Abl, murine), have shown Ab2 containing an internal image of JEV antigen.

MoAbs are frequently known to cross-react with two apparently dissimilar antigens. Many such unexpected cross-reactions have been reported with structural and cell surface proteins, DNA, haptens, phospholipids, lipopolysaccharides and proteoglycans (Crawford *et al.*, 1982; Jacob *et al.*, 1984; Rubin *et al.*, 1984; Serban *et al.*, 1985). Majority of such cross-reactions occur with MoAbs of IgM type having low affinity but high avidity due to their multivalency (Ghosh and Campbell, 1986). The cross-reactivty shown by MoAb NHA-1 of IgG2b subclass towards the histones indicates that these proteins (histones) might share a sequential homology with E glycoprotein of JEV. Using ALIGN programme (Dayhoff *et al.*, 1983), we have also recorded a homology between JEV E glycoprotein and calf thymus histones types 2A (slightly lysine-rich) and 4 (arginine-rich).

The anti-histone activity has been demonstrated in a number of diseases, e. g. systemic lupus erythematosus, rheumatoid arthritis (Fritzler and Tan, 1978; Tan, 1982; Rubin *et al.*, 1984). Gould *et al.* (1983) have shown an IgM type of antibody in mice early after JEV infection and in acute sera (IgM) collected from some of the human JE cases reacting both with the nuclei of uninfected/infected cells and with JEV antigen expressed in cytoplasm of the infected cells by indirect FA. We have tested paired and single serum samples (post-vaccination) from 9 human JE cases and 3 vaccinees (vaccinated with mouse brain JEV vaccine) respectively, obtained from South Arcot, Tamilnadu, India. Three paired sera, in addition to two convalescent serum samples reacted with the histones (type II-AS) by ELISA and with nuclei of infected/uninfected PS cells by indirecct FA. The serum samples from the three vaccinees did not react, either with histones or with the cell nuclei. In contrast, all these sera from clinical cases and vaccinees reacted invariably with JEV antigen by either test (data not shown). Portanova *et al.* (1988) have demonstrated a direct involvement of anti-histone antibodies in inducing spontaneous murine lupus. Thus the role of anti-histone antibodies in JE pathogenesis both in humans and in experimental animals, remains to be elucidated.

Acknowledgements. Authors are thankful to Dr. M. M. Gore for the generous help and invaluable suggestions, and Shri V. M. Ayachit for the technical help during the study.

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