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PREPARATION AND CHARACTERIZATION OF THE MONOCLONAL ANTIBODIES AGAINST JAPANESE ENCEPHALITIS VIRUS

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Summary. – Six mouse monoclonal antibodies (MoAbs) against Japanese encephalitis virus (JEV) were prepared and analyzed with indirect immunofluorescence assay (IFA), enzyme linked immunosorbent assay (ELISA), haemagglutination inhibition test (HI), neutralization test (NT), antibody dependent cell mediated cytotoxicity (ADCC) assay, antigenic site specific analysis and relative affinity measurement. These MoAbs could be divided into three classes by indirect immunofluorescence cross reactivity among four flaviviruses, $2H_4$, $2F_2$, and nG_2 were type specific; $2D_2$ and mC_3 were subgroup specific; and mG_9 was family specific. $2H_4$ and $2F_2$ had higher neutralization activity, $2D_2$ and mC_3 had the function of inducing ADCC effect, mG_9 had higher titer in HI. The six MoAbs recognized five antigenic sites on JEV envelope glycoprotein, $2H_4$ and $2F_2$ recognized the same or very similar antigenic site and their relative affinity was ranked as: $nG_2 > 2H_4 > 2D_2 > mG_9 > 2F_2 > mC_3$.

Key words: Japanese encephalitis virus; monoclonal antibody

Introduction

Flaviviruses display extensive serologic relatedness due to shared group specific antigens which link the members of the group; in general, cross reaction is most extensive in HI, intermediate in complement fixation and least in NT. Virus subgroups were formed in the basis of close antigenic ties, e.g., between members of tick-borne encephalitis virus complex (TBEV), or between some mosquito-borne viruses, such as St. Louis, Japanese, Murray Valley encephalitis and West Nile virus. Cross reactions both simplify and complicate serologic diagnosis of infections; use of one or two antigens in a test for antibody may suffice to detect a flaviviral infection, but extensive testing may be required to define the specific agent responsible. In areas where two or more viruses are

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endemic, superinfections produce broad anamnestic antibody reaction patterns which make specific serologic diagnosis, virus isolation and especially the molecular virological characterization extremely difficult (Monath, 1985). Furthermore, Japanese encephalitis is widely prevalent in Southeast Asia and the Western region of the Pacific Ocean with higher incidence, mortality and sequelae. Japanese encephalitis is severely threatening the health and life of human beings and there is no specific therapeutic way to treat it.

Studies have showed that it was very convenient to use MoAbs in viral antigenic analysis (Clarke, 1980; Trent, 1971; Roehrig *et al.*, 1982, 1983; Kobayashi *et al.*, 1985; Yewdell and Gerhard, 1981; Kimura-Kuroda and Yasui, 1983; Furuta, 1984; Heinz *et al.*, 1983; Schlesinger *et al.*, 1983; Grešíková and Sekeyová, 1984; Henchal *et al.*, 1982; Peiris *et al.*, 1982; Mathews and Roehrig, 1984) and early diagnosis (Xiu *et al.*, 1987; Bequan *et al.*, 1987). This investigation describes the preparation and characterization of the anti-JEV MoAbs and their use in antigenic analysis in order to find some clues for rapid diagnosis, protective subunit vaccine and effective ways for specific therapy.

Materials and Methods

Viruses. Several JEV strains were kindly provided by National Institute for the Control of Pharmaceutical and Biological Products in Peijing. Among them, Nakayama strain was isolated from a patient in 1936 in Japan, P_3 strain was isolated from a patient in 1949 in Peijing, SA_{14} strain was isolated from the mosquito in 1953 in Xian, JI strain was isolated from a pig in 1981 in Northeast China, and KT strain was isolated from a patient in 1953 in Guangdun. St. Louis encephalitis virus (SLEV), dengue type 2 virus (DENV), TBEV and Sindbis virus were kindly provided by the 5th Institute of the Military Medical Academy in Peijing. All these viruses were inoculated in the brains of suckling mice for three passages before use in present experiments. Their $TCID_{50}$ titer was assayed in BHK21 cell cultures. The stock virus was kept at -20 °C.

MoAbs and their F(ab')₂. Preparation of the anti-JEV MoAbs has been described by Xiu *et al.* (1985). The MoAbs $2H_2$, $2F_2$, mC_3, $2D_2$ and mG_6 were subclassified as IgG_{2a}, and the MoAb nG_2 belonged to IgG_{2b} subclass. MoAbs from the mouse ascitic fluids were purified by ammonium sulfate precipitation and DEAE-Sephadex A-50 (Pharmacial) ion exchange chromatography. The IgG of $2H_4$, $2F_2$ and mC_3 were mixed in equal parts, then digested with pepsin (Sigma) as described by Lamoyi and Nisonoff (1983). The $F(ab')_2$ was isolated with Sephadex G-150 (Pharmacial) chromatography, and was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Polyclonal antibody (PoAb) and normal mouse ascitic fluid. The PoAb was prepared by immunizing BALB/c mice with JEV-SA_{14}. The normal ascitic fluids were obtained from the BALB/c mice inoculated with Sp_2/O mouse myeloma cells.

ELISA. The indirect ELISA was done according to a procedure described by Kimura-Kuroda and Yasui (1983). Antibody titers were expressed as the final dilution of MoAb containing ascitic fluids giving 50 % of mean absorbancy value recorded in the virus control wells containing normal mouse ascitic fluids.

HI. HI was done by a half-micro method according to Hammon and Sather (1959). Haemagglutinin was prepared by the sucrose-acetone technique from the JEV infected suckling mouse brains. MoAb containing ascitic fluids were pretreated with acetone.

IFA. The antigen slides were prepared by dropping the JEV-SA_{14} infected BHK 21 cells (5×10^5/ml) onto them. The slides were fixed with cold acetone for 15 min washed with PBS and dryied. Each MoAb had to react with five different togaviruses to prove its specificity.

Neutralization test (NT). The neutralization activities of the MoAbs and their $F(ab')_2$ against JEV,

DENV, ELEV, TBEV and Sindbis virus were assayed in C6/36 *Aedes albopictus* cell cultures. The end point of the virus neutralization was defined as the reciprocal of the highest MoAb dilution capable to reduce the number of virus-infected cells by 50 %.

Antigenic analysis. The MoAbs were analyzed for whether they recognize the same antigenic site or not by the ELISA additivity test (Friquet *et al.*, 1983). The additive index (AI) was calculated for a pair of antibodies as follows:

$$AI = \left(\frac{2A_{1+2}}{A_1 + A_2} - 1\right) \times 100$$

where A_1, A_2 and A_{1+2} are the absorbancy values from the ELISA test, with the first antibody alone, the second antibody alone and the two antibodies together.

Measurement of the realtive affinity of the MoAbs. The measurement of the relative affinity of the MoAbs was performed according to Heyningen *et al.* (1983).

ADCC assay. The ADCC activities of the anti-JEV MoAbs have been reported (Zhang *et al.*, 1987). Normal or JEV infected mouse spleen cells were used as the effector cells and JEV infected BHK 21 cells were used as the target cells. The results were calculated according to Neguro *et al.* (1979).

Results

Biological character of the anti-JEV MoAbs

The immunological character of the anti-JEV MoAbs was quite heterogeneous; their IFA titers were 10^{-4} to 10^{-5}, ELISA titers 10^{-5} to 10^{-8}, mG_9 and mC_3 have higher titers in HI, mC_3 and $2D_2$ had the function of mediating ADCC effect; $2H_4$, $2F_2$ and mC_3 (especially the first two) have higher titers in NT; the mixed MoAbs had the highest neutralization activity. $F(ab')_2$ lost the HI activity, but had almost the same neutralization titer as the mixed MoAbs (Table 1). The

Table 1. Assays for the immunological activity of the MoAbs and their $F(ab')_2$

MoAbs	FA	ELISA	HI	NT	ADCC
$2H_4$	10^{-5}	10^{-8}	320	2×10^{-6}	-
$2F_2$	10^{-5}	10^{-8}	-	3×10^{-5}	-
mC_3	10^{-5}	10^{-8}	1 280	5×10^{-5}	10^{-2}
mG_9	10^{-5}	10^{-8}	2 560	-	-
$2D_2$	10^{-5}	10^{-5}	-	-	
nC_2	10^{-4}	10^{-4}	-	-	-
M-MoAb	10^{-5}	10^{-8}	320	1×10^{-7}	10^{-1}
$F(ab')_2$	NT	NT	-	1×10^{-6}	-
PoAb	10^{-3}	10^{-6}	320	5×10^{-3}	10^{-2}
$Sp_2/0$	-	-	-	-	-

-: no activity
NT: not tested

Table 2. Specificity and reaction types of the MoAbs

MoAbs	IFA					HI					NT				
	JEV	SLEV	TBEV	DENV	Sindbis	JEV	SLEV	TBEV	DENV	Sindbis	JEV	SLEV	TBEV	DENV	Sindbis
$2H_4$	20489	< 10	< 10	< 10	< 10	320	< 20	< 20	< 20	< 20	250000	< 20	< 20	< 20	< 20
$2F_2$	20480	< 10	< 10	< 10	< 10	< 20	NT	NT	NT	NT	320000	< 20	< 20	< 20	< 20
nG_2	5120	< 10	< 10	< 10	< 10	< 20	NT	NT	NT	NT	< 20	NT	NT	NT	NT
mC_3	20480	20	< 10	< 10	< 10	2560	20	< 20	< 20	< 20	450	< 20	< 20	< 20	< 20
$2D_2$	20480	5120	< 10	< 10	< 10	< 20	NT	NT	NT	NT	< 20	NT	NT	NT	NT
mG_9	20480	20480	5120	5120	< 10	20480	10240	5120	10240	< 20	< 20	NT	NT	NT	NT
MAJEV	320	80	80	320	< 10	320	160	40	40	< 20	320	< 20	< 20	< 20	< 20
Sp_2/O	< 10	< 10	< 10	< 10	< 10	< 20	< 20	< 20	< 20	< 20	< 20	NT	NT	NT	NT

NT: not tested

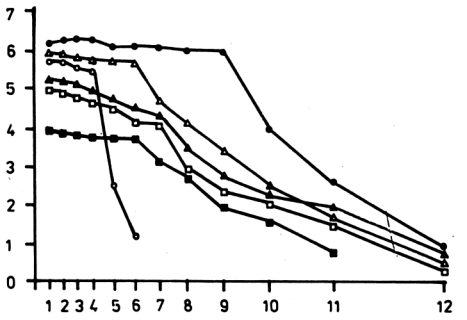

Fig. 1
Measurement of relative affinities of the MoAbs
● - nG_2; △ - $2H_4$; ▲ - $2D_2$; □ - mG_9; ■ - $2F_2$; ○ - mC_3
Abscissa: Lg of MoAb concentration (with initial of 15 mg/ml). Ordinate: Ratio of OD value of MoAb to normal mouse ascitic gluid (P/N).

relative affinity of the six MoAbs was $nG_2>2H_4>2D_2>mG_9>2F_2>mC_3$, in the concentration range of 0.01 to 1.5 ng (Fig. 1).

Specificity of the antiJEV MoAbs

In order to determine the specificity of the anti-JEV MoAbs, SLEV, TVEV, DENV and Sindbis virus were assayed in IFA for cross reactions. Using these results, we could divide these six MoAbs into three classes. $2H_4$, $2F_2$ and nG_2 were type specific, they reacted only with JEV. mC_3 and $2D_2$ were subgroup specific, they could react with SLEV as well as JEV. mG_9 was family specific, it could react with all four flaviviruses but could not react with Sindbis virus (alphavirus) (Table 2). The details of this study were described by Xiu *et al.* (1985).

The additive index of $2H_4$ and $2F_2$ showed that they recognized the same or very similar antigenic site, while the other MoAbs bound at different sites (Table 3).

Discussion

Antigenic determinants on the surface of JEV have been studied for a long time. It was known that JEV has three kinds of structural proteins: envelope,

Table 3. ELISA additivity test of the MoAbs

Ascitic fluids (dilutions)	AI (%)				
	$2H_4$ (10^{-6})	$2F_2$ (10^{-5})	mC_3 (10^{-3})	SD_2 (10^{-5})	nG_2 (10^{-8})
$2F_2$ (10^{-5})	23.51				
mC_3 (10^{-3})	65.83	68.00			
$2D_2$ (10^{-5})	74.77	100.00	91.76		
nG_2 (10^{-8})	73.38	78.90	104.42	77.03	
mG_9 (10^{-5})	60.64	76.92	83.76	81.82	53.19

glycoprotein (gP), capsid protein and membrane protein. Only the envelope gP was related with the infectivity (Takegami *et al.*, 1982). Trent (1977) found by isoelectric focusing and radioimmunoassay that there are three antigenic determinants on the flavivirus gP. They are type specific, subgroup specific and family specific. This was first confirmed by Clarke (1980) with antibody absorption and HI, and then also with MoAbs through HI and IFA (Kimura-Kuruda and Yasui, 1983; Roehrig *et al.*, 1983; Heing *et al.*, 1983). In this study, the cross IFA showed that $2H_4$, $2F_2$ and nG_2 are type specific because they were reactive only with JEV; mC_3 and $2D_2$ are subgroup specific because they were reactive with SLEV besides JEV in the same subgroup; mG_9 could react with JEV, SLEV, TBEV and DENV, so it is flavivirus specific MoAb. The six MoAbs are directed against antigens on the envelope gP. The results also uncovered that antigenic determinants on the surface of JEV can be divided further. According to different reactivities, the type specific antigenic determinants correspond to three epitopes. One, corresponding to $2H_4$ participated in HI and neutralization; another corresponding to $2F_2$ participated in neutralization; the third, corresponding to nG_2 did not show any biological function. The subgroup specific antigenic determinants can be divided into two epitopes. One (mC_3) participated in HI, neutralization, and had higher activity of ADCC; another ($2D_2$) only had lower titer in ADCC. The family specific antigenic determinant has at least one (mG_9) epitope which participated in HI but not in neutralization, while according to the ELISA additivity test of the MoAbs, the antigenic determinants recognized by $2H_4$ and $2F_2$ were almost identical. Thus we can assume that there are at least five antigenic determinants on the surface of JEV particle. This is consistent with the data reported by Kimura-Kuroda and Yasui (1983).

The biological functions of the antigenic determinants on the envelope gP of JEV in our experiments were quite heterogeneous. ADCC activities were related to the subgroup specific antigenic determinants can be further divided. There are at least three kinds of antigenic determinants controlling the neutralization activity. One determinant controls neutralization only; another controls both neutralization and HI; the third is related to ADCC apart from neutralization and HI. This phenomenon has been observed also in other flaviviruses. Kimura-Kuroda and Yasui (1983) reported that infection and haemagglutination are controlled by different antigenic determinants, but Roehrig *et al.* (1983), consided that the two functions were controlled by the same antigenic determinant. According to the studies of Heinz *et al.* (1983) the mentioned phenomenon could be explained by the allosteric effect of protein. The functions of an antigenic determinant were determined by the changes taking place after binding of the MoAb (Atassi, 1980).

The MoAbs have been very useful in the virological research. In the present study, the relative affinity of the six MoAbs was quite different. The IgG_2b subclass MoAb nG_2 did not have any other significant biological activity except the highest affinity with type specificity so it can be considered as very valuable in immunological assays. $2H_4$ is another JEV type specific MoAb, it had not only

higher affinity, but many biological functions also such as haemagglutination inhibition and neutralization. Therefore, $2H_4$ MoAb is not only a good reagent for *in vitro* immunoassays, but an even better product for *in vivo* treatment. Further studies on the antigenic structures recognized by $2H_4$ MoAb would provide us a valuable information for the preparation of the subunit JEV vaccine.

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