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A RAPID METHOD FOR DETECTION OF FLAVIVIRUS ANTIGENS: STAPHYLOCOCCAL CO-AGGLUTINATION TEST USING MONOCLONAL ANTIBODIES TO JAPANESE ENCEPHALITIS VIRUS

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Summary. — *Staphylococcus aureus* rich in protein A when coated with monoclonal antibodies (MoAb) to Japanese encephalitis virus (JEV) gave a highly specific reaction with flavivirus antigens. The bacteria coated with JEV species-specific MoAb gave a strong co-agglutination with fifty-six JEV isolates from various parts of China, but no co-agglutination with Murray Valley encephalitis (MVE) and Kunjin (Kun) virus antigens. The flavivirus- and subgroup-specific MoAbs were reactive with MVE and Kun, as well as with the majority of the JEV strains. Blocking test with homologous MoAbs abolished co-agglutination further confirming its specificity. Numerous virus particles were observed on the surface of MoAb-coated staphylococci under the electron microscope after co-agglutination. The test appeared rapid, specific, simple to perform, and useful for rapid detection and identification of flaviviruses.

Key words: *bacterial co-agglutination*; *monoclonal antibodies*; *Japanese encephalitis virus*; *flaviviruses*

Introduction

Rapid detection of viral antigens has been achieved by staphylococcal co-agglutination test, such as detection of avian viruses (Pandya *et al.*, 1981), calf diarrhea rotavirus (Skaug *et al.*, 1983), and turkey rotavirus (Kang *et al.*, 1985). More recently, we have been successful in detection of the adult diarrhea rotavirus directly in the faecal samples by the co-agglutination test, and the specificity of the test has been well confirmed (Zhang *et al.*, 1988).

In the area of arboviruses, bacterial co-agglutination test was performed on dengue viruses using polyclonal immune ascitic fluids (IAFs) for coating of the bacteria (Huang *et al.*, 1983; Chen, 1984). When we attempted to coat

the bacteria with monoclonal antibodies to JEV, satisfactory results were obtained for rapid detection of JEV antigens as well as the antigens of other flaviviruses. Optimalization of the test is described in the present paper.

Materials and Methods

Viruses. Fifty-six JEV isolates from various parts of China were used. Among them four strains were the prototypes, including Peking (A_2), P_3, SA_{14}, and Gao strains of JEV. Murray Valley encephalitis strain WK 65 and Kunjin strain OR 393 viruses were also included for comparative studies.

MoAbs to JEV. The MoAbs were prepared as described previously (Xu *et al.*, 1985), including JEV species-specific clones 2F2 and 2H4, directing against M protein of the virion, subgroups directed against C protein, and flavivirus-specific clone mG9, directed against E protein. Another flavivirus-specific MoAb to JEV No. 301 was kindly supplied by Dr. K. Yasui, also directed against E protein (Kimura-Kuroda and Yasui, 1983). All the MoAbs were of IgG_{2a} class. Details including their antibody titres are listed in Table 1. Sp 2/0 or normal ascitic fluids were used as controls.

Staphylococcus aureus Cowan I bacteria. Inactivated and lyophilized staphylococcal bacteria were purchased from the Shanghai Institute of Biological Products, Shanghai.

Coating of staphylococcal bacteria with MoAbs. The methods have been described previously (Zhang *et al.*, 1988), with minor modifications. Briefly, the bacteria were washed once with normal saline and centrifuged at 5,000 rev/min for 5 min after reconstitution. A 50 % bacterial suspension was made up by adding an equal volume of normal saline to the packed bacteria and mixing. Equal volume of MoAbs at an adequate dilution was mixed with the 50 % bacterial suspension and incubated at 37 °C for 30 min at occasional mixing. Additional three washings were performed to remove free antibodies. The 50 % suspension of coated bacteria was stored until use 0.02 % sodium azide was added.

Bacterial co-agglutination test. A 2 % suspension of coated bacteria was made up in 0.01 mol/l phosphate buffered saline (PBS), pH 7.4 with 0.5 % Tween 20, and sodium azide was also added when required. 10 μl of viral antigen was mixed with 10 μl of 2 % bacteria suspension in a marked circle on a slide by swirling for 3–5 min until agglutination formed. Normal mouse brain antigen and the bacteria-coated with Sp 2/0 or normal ascitic fluids were used as controls. The agglutination patterns were judged by the naked eye and/or under a microscope at a low magnification and were classified from strong to slight and no agglutination (++++, +++, ++, + and −),

Electron microscopic observations. The MoAbs used for coating of the bacteria for this purpose were JEV-specific 2F2 plus 2H4, and Sp 2/0 fluid was used as controls. The virus preparation was from JEV strain P_3-infected C6/36 cell culture, and the culture fluid was 50X concentrated by polyethylene glycol precipitation (Igarashi *et al.*, 1973). After incubation of MoAb-coated bacteria with the concentrated JEV preparation at room temperature for 20 min and centrifuged at 5,000 rev/min for 3 min, the resuspended bacteria were observed by electron microscopy with phosphotungstic staining (Brenner and Horne, 1959).

Abbreviations: MoAb, Monoclonal antibody; JEV, Japanese encephalitis virus; MVE, Murray Valley encephalitis; Kun, Kunjin; IAF, Immune ascitic fluid; PBS, Phosphate buffered saline; ELISA, Enzyme-linked immunosorbent assay

Results

Conditions of bacterial co-agglutination test with JEV antigens

As shown in Table 2, a thirty-minute incubation at 36 °C gave maximal sensitization of the bacteria with JEV-specific MoAbs.

As shown in Table 3, the buffered saline at pH 6.0 gave a very low co-agglutination titre. Both pH 7.0 and 8.0 appeared to be optimal for giving the highest titres, whereas pH 9.0 and normal saline gave suboptimal results.

Table 1. Antibody titres of MoAbs assayed by neutralization haemagglutination inhibition and immunofluorescence tests

Clone	Specificity	Directed against	Reciprocal antibody titre**			Reference
			NT	HI	IFA	
2F2	JEV species	M	320 000	<20	81 920	Xu *et al.*, 1985
2H4	JEV species	M	250 000	320	20 480	Xu *et al.*, 1985
2D2	Subgroup	C	<20	<20	20 480	Xu *et al.*, 1985
mG9	Flavivirus	E	<20	20 480	20 480	Xu *et al.*, 1985
301*	Flavivirus	E	<50*	160 000	Not done	Kimura-Kuroda and Yasui, 1983

** Assayed against the homologous JEV strain i.e. strain SA_{14} for MoAbs 2F2, 2H4, 2D2 and mG9, and strain JaGAr-01 for MoAb 301

* MoAb 301 had an ELISA (Enzyme-linked immunosorbent assay) titre 2.7×10^7

* Plaque reduction neutralization titre

Specificity of bacterial co-agglutination test

Staphylococci coated with JEV-specific MoAbs, either 2F2 or 2H4, always gave strong co-agglutination, when rescreening the antigens of fifty-six JEV strains from various parts of China, including from North-East, North-West, East and South of the country. All these isolates had been previously identified as JEV by a routine test. As shown in Table 4, with five JEV strains as representatives, in comparison to the other flaviviruses MVE and Kun, JEV-specific MoAbs 2F2 and 2H4 reacted strongly with JEV only, but were not cross-reactive with MVE and Kun viruses, whereas the subgroup-specific 2D2, and the flavivirus-specific mG9 and No. 301 MoAbs

Table 2. Incubation time required for coating of staphylococci with JEV-specific MoAbs 2F2 plus 2H4

Incubation time (min)	Reciprocal JEV antigen dilution									Normal antigen control (1 : 1)
	1	2	4	8	16	32	64	128	256	
5	++++	+++	++	+	+	−	−	−		−
10	++++	++++	++++	+++	++	+	−	−		−
20	++++	++++	++++	++++	+++	+	−	−		−
30	++++	++++	++++	++++	+++	++	+	−		−
40	++++	++++	++++	++++	+++	++	+	−	−	−
60	++++	++++	++++	++++	+++	++	+	−	−	−

Incubation at 36 °C

Table 3. Effects of pH on staphylococcal co-agglutination with JEV antigen

pH of the buffer*	Co-agglutination with dilutions of JEV antigen								Controls	
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	Normal antigen	Buffer
6.0	++++	++	+	±	±	±	±	−	−	−
7.0	++++	++++	++++	+++	++	+	+	−	−	−
8.0	++++	++++	++++	+++	++	+	−	−	−	−
9.0	++++	++++	+++	++	+	−	−	−	−	−
Normal saline	++++	++++	+++	++	+	±	−	−	−	−

* Both JEV (Gao strain) antigen and bacterial suspension were diluted with buffered saline at the same pH levels, with citric acid-phosphate buffered saline for pH 6−8, and borate buffered saline for pH 9.0

Table 4. Co-agglutination of flavivirus antigens with staphylococci coated with MoAbs to JEV of different specificities

Virus antigen	MoAb of specificities to					Normal ascitic fluid control
	Species		Subgroup	Flavivirus		
	2F2	2H4	2D2	mG9	301	
JEV						
A_2	++++	++++	+	−	−	−
P_3	++++	++++	+	−	−	−
Shanghai-1	++++	++++	++	−	−	−
SA_{14}	++++	++++	+++	++	++	−
Gao	++++	++++	+++	+++	+++	−
MVE	−	−	++++	+++	+++	−
Kun	−	−	++++	++	++	−
Normal antigen	−	−	−	−	−	−

were crossreactive with MVE and Kun viruses. A peculiar phenomenon observed was that the two flavivirus-specific MoAbs mG9 and No. 301 did not react with A_2, P_3, and Shanghai-1 strains at all, but reacted well with SA_{14} and Gao strains of JEV (Table 4), as well as with the rest of fifty-three JEV strains (data not shown).

Blocking test

In order to confirm the specificity of the bacterial co-agglutination test, blocking test was performed using JEV-specific MoAbs 2F2 or 2H4 at a dilution of 1 : 16 for the treatment of an equal volume of JEV antigens at 4 °C overnight. Then the treated antigens were tested for co-agglutination

Table 5. Blocking test for staphylococcal co-agglutination using JEV-specific monoclonal antibodies for coating and blocking

Blocking MoAb	Co-agglutination of 2F2 plus 2H4 MoAb-coated bacteria with blocked JEV antigen							
	A_2		P_3		SA_{14}		GAO	
	1 : 2*	1 : 4	1 : 8	1 : 16	1 : 2	1 : 4	1 : 4	1 : 8
Sp 2/0	+++	++	++++	+++	+++	++	+++	++
2F2	±	−	−	−	−	−	−	−
2H4	−	−	−	−	−	−	−	−

* Final dilutions of the antigens

with JEV-specific MoAb-coated bacteria, in which 2F2 plus 2H4 were used. As shown in Table 5, co-agglutination by the antigens of these four JEV strains was blocked by the homologous JEV-specific MoAbs 2F2 or 2H4, whereas the control ascitic fluid Sp 2/0 had no blocking activity.

Bacterial co-agglutination with JEV observed under electron microscope

Numerous JEV particles adhered to the surface of MoAb-coated bacteria cells, forming a monolayer (Fig. 1). Only very rare virus particles randomly adhered to the surface of Sp 2/0-coated bacteria cells after the same treatment. No virus particle was seen on the surface of specific MoAb-coated bacterial cells when incubated with uninfected (control) C6/36 cell culture fluid (not shown).

Discussion

Polyclonal antibodies were used for coating of staphylococci in the co-agglutination test for virus detection in the previous studies (Pandya *et al.*, 1981; Skaug *et al.*, 1983; Kang *et al.*, 1985; Huang *et al.*, 1983; Chen, 1984; Zhang *et al.*, 1988). In the present study, however, MoAbs to JEV with different specificities were used for coating of the bacteria. The specificities of the MoAbs, i.e. their species-, subgroup- or flavivirus-specificity had been proved by neutralization, immunofluorescence and haemagglutination inhibition tests (Xu *et al.*, 1985; Kimura-Kuroda and Yasui, 1983). The bacterial co-agglutination test showed similar specificities of these MoAbs as shown in Table 4. The specificity of the co-agglutination test was also confirmed by the blocking test using anyone of the homologous MoAbs (Table 5). Furthermore, as seen in Table 1, not all the MoAbs were reactive with JEV in neutralization and haemagglutination tests, even when tested with homologous JEV strains.

Bacterial co-agglutination is likely to occur due to antigen-antibody binding, its optimal pH range appeared to be much broader as that of haemagglutination test, probably as result of a different mechanism in binding of virus particles. Since the buffered saline at pH 7.0 and 8.0 gave the most optimal results for the bacterial co-agglutination with JEV antigens, it should be convenient for us to use 0.01 mol/l PBS, pH 7.4, the same as that used in the enzyme-linked immunosorbent assay (ELISA).

Among the fifty-six JEV strains tested, three strains including A_2, P_3, and Shanghai-1 strains did not react with the fllavivirus-specific MoAbs mG9 and No. 301 in the co-agglutination test (Table 4). It is worthwhile to notice that these two flavivirus-specific MoAbs were coming from two different laboratories, and furthermore, the experiment had been repeated many times for confirmation. This phenomenon might be due to characteristics of strain differences rather than due to the "threshold sensitivity" of the test, since the non-reactivity of these three JEV strains with flavivirus-specific MoAbs was obtained when undiluted antigens were used in the test. In contrast, positive results were obtained in bacterial co-agglutination reaction between these two EoAbs and the rest of JEV strains as well as

MVE and Kun viruses. The phenomenon remains to be elucidated in further work. However, that might not interfere with the use of JEV species-specific MoAbs, which always reacted with JEV antigens very well, for diagnostic purpose.

As observed under electron microscope, numerous JEV particles could be seen adherent to the surface of MoAb-coated bacteria cells when they strongly co-agglutinated with a concentrated JEV preparation from the C6/36 cell culture, but no virus could be seen between the two adjacent agglutinating cells. Which makes the MoAb-coated bacteria to agglutinate, the intact virion or disrupted viral component? The question remains to be elucidated. However, this approach appears useful for trapping of virus particles from a virus suspension as an easy way for recognizing the viral particles specifically under electron microscope.

Bacterial co-agglutination test appears to be a simple, rapid, specific and economic method for detection of flavivirus antigens. Viral antigens from either infected mouse brain or C6/36 cells were detectable by the test. The method has been proved useful for rapid detection and identification of JEV isolates. The blocking test for co-agglutination not only showed its specificity, but also appeared to be applicable to antibody detection by the test. More recently, using the co-agglutination test, rapid detection of JEV antigens in the eluted fractions from hydroxylapatite column chromatography for purification of virus preparations made it possible to pool the virus-containing fractions soon after their collection. The fractions positive in co-agglutination were in agreement with the results of ELISA.

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Explanation of Figure (Plate VIII):

Fig. 1. Staphyloccocal co-agglutination with JEV particles under electron microscope (magn. 93 000×). The bacteria were coated with JEV-specific MoAbs, 2F2 plus 2114. The preparation from infected C6/36 cell culture fluid was concentrated 50×.