The Analysis on Antigenic Determinants of Japanese Encephalitis Virus with Reference to the Monoclonal Antibody

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Abstract: An attempt to analyse the antigenic determinants of Japanese encephalitis (JE) virus by the application of monoclonal antibody method was made in this study. The hybrid cell culture was prepared by fusion of non-secreting myeloma cells and spleen cells immunized with JE virus vaccine. The hybrid cells were screened for the antibody production against JE virus by the enzyme-linked immunosorbent assay (ELISA). At least, nine of cloned cell lines were able to detect the antibody production against the antigen of subgroup specific determinants.

INTRODUCTION

Since Köhler and Milstein (1975) reported that the continous cell lines producing the homogenous antibody were able to prepare by the fusion of mouse myeloma cells to spleen cells derived from immunized doner, it is paid the attention that the analysis of antigenic determinants of viruses and the large amount of antibody production of a desired specificity might be provided by the application of hybridoma cell lines mentioned above. An antibody produced by the hybridoma cell is called the monoclonal antibody which indicates the advantage to overcome the classical way to prepare the antiserum with immunized animals. The monoclonal antibodies against numerous antigens have been provided successfully, including antibodies against lymphocytes and other cell surface antigens (Hammerling, 1977; Trucco, *et al.*, 1978; Köhler *et al.*, 1977), human leucocytes antigen (Brodsky, *et al.*, 1979), tumor antigen (Ritz *et al.*, 1980; Steplewski, *et al.*, 1979, 1980), 2,4,6-trinitrophenol (TNP) fowl gammaglobulin (Köhler, *et al.*, 1976) as well as influenza virus (Koprowski, *et al.*, 1977; Gerhard, *et al.*, 1975, 1976, 1978; Van Wyke, *et al.*, 1980; Yewdell, *et al.*, 1979), reovirus (Hayes, *et al.*, 1981; Lee, *et al.*, 1979), dengue virus (Dittmar, *et al.*, 1980), rabies virus (Wiktor, *et al.*,

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1978; Flamand, *et al.*, 1980), SV40 (Martinis, *et al.*, 1978; Gurney, *et al.*, 1980) and malaria parasite (Yoshida, *et al.*, 1980).

The reports on antivirus monoclonal antibodies indicated that the great contributions have been developed in the study on the distinct antigenic relationship among viruses and the mode of infection with reference to the analysis of viral antigens. Gerhard (1976) and Webster *et al.*, (1981) demonstrated the anigenic varitability of influenza viruses using the monoclonal antibody against the hemagglutinin. Further studies on the antigenic drift in the hemagglutinin glycoproteins of influenza viruses by means of monoclonal antibodies revealed the informations about the frequency of mutation in the antigenic sites and the number of non-overlapping antigenic determinants which influence the identification and typing of different strains among influenza viruses. On the other hand, so far, the study on the monoclonal antibody against SV40 virus presented possible existence of the major tumor antigen in the immunologically distinguishable subclasses and demonstrated the role of an antigenic determinant in the minor tumor antigen of several mammalian species (Gurney, *et al.*, 1980). The reovirus monoclonal antibody was applied to detect the localization of viral proteins in the virion (Lee *et al.*, 1981) and to identify the specific receptor on the host cell (Hayes, *et al.*, 1981). Recently, attempts to clarify the antigenic determinant of rabies virus and malaria parasite, which cause the important diseases in man in tropical areas, were made for the improvement of vaccination with reference to the application of monoclonal antibodies against the virus and the parasite (Wiktor, *et al.*, 1980; Potcnjak, *et al.*, 1980).

These studies mentioned above indicate that the monoclonal antibodies are not only available to analyse the antigenicity of viruses but also to be applied as reagents for the direct identification of causative agents without depending neutralization, complement fixation and hemagglutination inhibition tests.

Since the production of monoclonal antibody against Japanese encephalitis virus has not been reported previously, it is undertaken in this study. The Japanese encephalitis virus belongs to flaviviruses (group B arboviruses) which are classified into three immuno-subtyping by the antibody absorption method (Okuno, *et al.*, 1968). Upto the present, the immuno-subtyping of different strains of Japanese encephalitis virus isolated has not definitely been confirmed yet. However, some reports suggest a minor antigenic difference between classicl and a current strains by the kinetics analyss in the antibody absorption method (Okuno, *et al.*, 1968). Furthermore, the identification of viruses isolated is still carried out by the neutralization, hemagglutination inhibition and complement fixation tests, which are considered to be complicated and not favourable techniques. In this study, an attempt was made to analyse the antigenic determinants of Japanese encephalitis virus and to improve the identification procedure of the strains isolated with reference to the monoclonal antibody against the virus.

MATERIALS AND METHODS

Antigens:

The formalin inactivated and purified Japanese encephalitis (JE) virus preparation was kindly obtained from the Kanonji Institute, Research Foundation for Microbial Disease, Osaka University.

Myeloma cells:

Three types of myeloma cell lines; P3X63-Ag8/653, NS-1 and SP-2/0-Ag14 were obtained from Salk Institute, USA, Research Institute for Microbial Diseases, Osaka University and Oita Medical College, respectively. These cell lines lack the enzyme of hypoxanthin-guanine phosphoribosyl transferase (HGRT) and they are killed in the hypoxanthin, aminopterine and thymidine (HAT) selective medium. The cells were grown in the Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with gentamycine and ten percent fetal calf serum and they were maintained at 37°C in hnmidified atmosphere at five percent of CO_2.

Imunization of mice and preparation of spleen cells:

Five or four weeks old Balb/C mice, which have identical histocompatibility antigen to the myeloma cells, were injected subcutaneously with one ml of ten-fold diluted JE vaccine mixed with epuivalent volume of imcomplete Freund's adjuvant. The second and the third immunization were given intraperitoneally with one ml of undiluted vaccine, two and four weeks later respectively. On the third day after the last injection, the spleens were removed, placed on a stainless steel mesh, minced with scissor and forced to pass through the mesh into a beaker supplied with DMEM solution using a plunger of a disposable two ml syringe. The resulting spleen cell suspension was spun down at 1,200 rpm for ten minutes. The supernatant was removed the pellet was tapped gently and suspended in ten ml of cold 0.17 M of NH_4Cl solution for ten minutes to lyse the erythrocytes. After washed twice, the number and the viability of spleen cells were determined by trypan blue exclusion and the number of spleen cells was adjusted to 1×10^8 cells per ml.

Fusion of myeloma cells to spleen cells:

The myeloma cells were washed once with the serum free DMEM solution, and a suspension of 10^7 myeloma cells per ml in the DMEM spplemented with ten percent fetal calf serum was mixed with an equivalent volume of spleen cell suspension. The co-pellet was formed by the centrifugation at 1,200 rpm for ten minutes and the supernatant was poured off and the tube was kept and inverted until the remaining liquid was removed by sucktion. The myeloma cells and spleen cells were fused with polyethylen glycol (PEG) nsing a modified method described by Galfer (1977). A volume of 0.8 ml of PEG (Mw 6,000, Koch Light Lad. Ltd. England) dissolved at fifty percent in the DMEM solution was added dropwise to the cells over one minute. The cell

suspension was mixed by occasional agitation for an additional one minute follwed by dilution with two ml of DMEM solution over a period of two minutes. Eight ml of DMEM solution was added during the next three minutes while the tube was shaken every few seconds. The cells were pelleted by the centrifugation at 1,000 rpm for five minutes. The cell pellet was resuspended carefully with DMEM solution containing twenty percent fetal calf serum and the cells were plated into 24 wells tissue culture plates so that the density of plated cells should not exceed 1 to 2×10^6 spleen cells per a well.

Selection of hybridoma cells and screening of antibody production:

On the second day of hybridoma cell culture, half amount of medium was removed and replaced with an equivalent volume of DMEM solution supplemented with twenty percent fetal calf serum and double concentrated HAT. The medium was changed every three to four days wth HAT-DMEM. On the seventh to tenth day later, the spleen cells usually died out in the tissue culture medium automatically and the development of hybrid colonies could be observed. The culture fluid in each well was assayed for the antibody production by enzyme-linked immunosorbent assay(ELISA), while the colour of medium turn to change to yellow. The HAT medium was replaced into DMEM medium containing twenty percent fetal calf serum and the cells were transfered into flasks for further development of cell culture which was used for cloning and product analysis.

Screening for antibody producing hybridoma clones by the ELISA:

The antibody against JE virus antigen was detected by the ELISA which was described previously (Igarashi, *et al.*, 1981). The plastic microplate (U type, Sanko Pure Chemical Co. Tokyo) was coated with 100 microliter of JE virus vaccine per a well. The plate was incubated in a refrigerator overnight and washed three times for each three minutes. The fluid of hybridoma cell culture was applied to the well and incubated at 37 C. The fluid was removed and the plate was washed. The peroxidase conjugated anti-mouse immunoglobulin diluted at 1 : 400 was added and the plate was incubated at 37 C for one hour. Then, the wells were washed and 100 microliter of substrate solution was added to each well. The reaction was stopped after fourty minutes by the addition of 75 microliter of 4N of H_2SO_4 solution to each well. The optical density at 500 nm wave length of the reactant in each well was recorded by the measurement of Corona double beam spectrophotometer. The optical density which showed more than twice the value in the control fluid of myeloma cell culture alone was judged as the positive reaction.

Cloning of antibody producing hybridoma cells:

It is desirable to clone the hybridoma cells as soon as possible, when the antibody production was detected. The limitting dilution method for the selection of clone was used. Each small aliquotes in which might contain one cell on average were dispensed into 96 well plate. The growth of hybridoma cell was assisted by the co-cultivation

with the feeder layer of peritoneal macrophages using 10^5 cells per a well, and the medium was changed every three days. When the fluid in the wells showed the antibody production by the ELISA, the cells were transfered to 24 well plates and eventually to the flasks.

Production of antibody derived from cloned cell line:

The antibody producing cloned cells were maintained by serial passage in the culture flask and stored occasionally in the liquid nitrogen. Otherwise, the cloned cells were inoculated into mice intraperitoneally to maintain in the condition of solid or ascites fluid. For the production of antibody, the four weeks old mice were treated with intraperitoneal inoculation of 0.5 ml of pristane (2,6,10,14-tetra-methylpentadecane) one week prior to an intraperitoneal transference of more than 10^6 hybridoma cloned cells per mouse. The ascites fluid and solid tumor were harvested on the tenth to the fourteenth day after the transference of hybridoma cloned cells. The ascites fluid was spun down to remove the cells and tested for the production of antibody against JE virus antigen. The cells sedimented were transfered to aditional mice, otherwise, they were stored in the liquid nitrogen.

Immunodiffusion test:

The Ouchterloney method was used for the identification on immunoglobulin class in the hybridoma serum and ascites fluid. The rabbit antisera afainst mouse immunoglobulin G; IgG2a, IgG2b, IgG3, λ and κ light chains were obtained from the Miles Laboratory. The agar plate was prepared with wells, 2 mm in diameter, for application of the specimens. To the center well was placed the hybridoma serum or the ascites fluid and the surrounding wells were filled with each specific anti-immunoglobulins. After 24 or 36 hours incubation in the refrigerator, the precipitin lines were observed.

Neutralization test:

The mixture of ten-fold serial dilution of virus preparation and the constant amount of ascites fluid was incubated at 37 C for one and half hours and inoculated either into BHK cell cultures or into each mouse intracerebrally. The mortality was recorded and LD_{50} was calculated by Reed and Muench method.

Challenge test:

The costant amount of ascites fluid (0.6 ml) was inoculated into mice intraperitoneally and six hours later, the mice were challenged intracerebrally by 0.01 ml of virus suspension (10^7 LD_{50}/ml).

RESULT

Frequency of hybridization and production of antibody in culture cells:

The results of four fusion experiments performed with spleen cells of Balb/C mice immunized with JE virus vaccine and three different lines of myeloma cells; 63-Ag8/653, SP-2 and NS-1 strains are summarized in Table 1. Among these myeloma cell lines, the fusions between NS-1 strain and immunized spleen cells yielded twenty hybridomas in mass cultures. Five of them indicated antibody production against JE virus antigen by the ELISA. On the other hand, the clones secreting antibody derived from 63-Ag8/653 myeloma cells were not able to isolate. The six clones out of fourty mass cultures derived from SP-2 myeloma cells did not grow to mass culture due to some unfavourable conditions (Table 1).

Isolation of clones producing anti-JE antibody:

Five mass cultures, which constantly produced antibody in culture fluids, were cloned by the limitting dilution method. As shown in Table 2, some single cell clones indicated the production of anti-JE antibody by the ELISA. Eight of the clones were grown to produce tumors in Balb/C mice. The ascites and the serum derived from mice transplanted the hybridoma cells contained higher titer of antibody against JE virus antigen than those in fluids obtained from the tissue culture of hybridoma clone cells (Table 2, Figure 1).

Table 1. Frequency of hybrid and antibody producing cultures

Fusion No.	Myeloma cell line	No. of wells with cell outgrowth / No. of wells seeded	No. of wells with anti-JE antibody / No. of wells with cell outgrowth
1	X63−Ag8.653	10/96	0/10
2	X63−Ag8.653	17/72	0/17
3	SP−2	40/68	6/40
4	NS−1	20/48	5/20

Table 2. ELISA test against JE antigen using culture medium from single cell clone

Clone No.	ELISA OD range of triplicate tests	Production of ascites in Balb/c mice
1	0.449−0.526	0
2	0.370−0.470	
4	0.386−0.536	0
5	0.378−0.520	0
6	0.394−0.520	
7	0.374−0.524	
8	0.370−0.520	
10	0.383−0.519	
11	0.396−0.480	0
12	0.357−0.514	0
13	0.335−0.498	0
14	0.350−0.513	0
15	0.366−0.509	
18	0.397−0.491	
20	0.390−0.506	0
21	0.359−0.492	0
23	0.414−0.449	
24	0.371−0.417	
25	0.458−0.506	
32	0.375−0.455	
33	0.335−0.458	
Cont.	0.018−0.037	

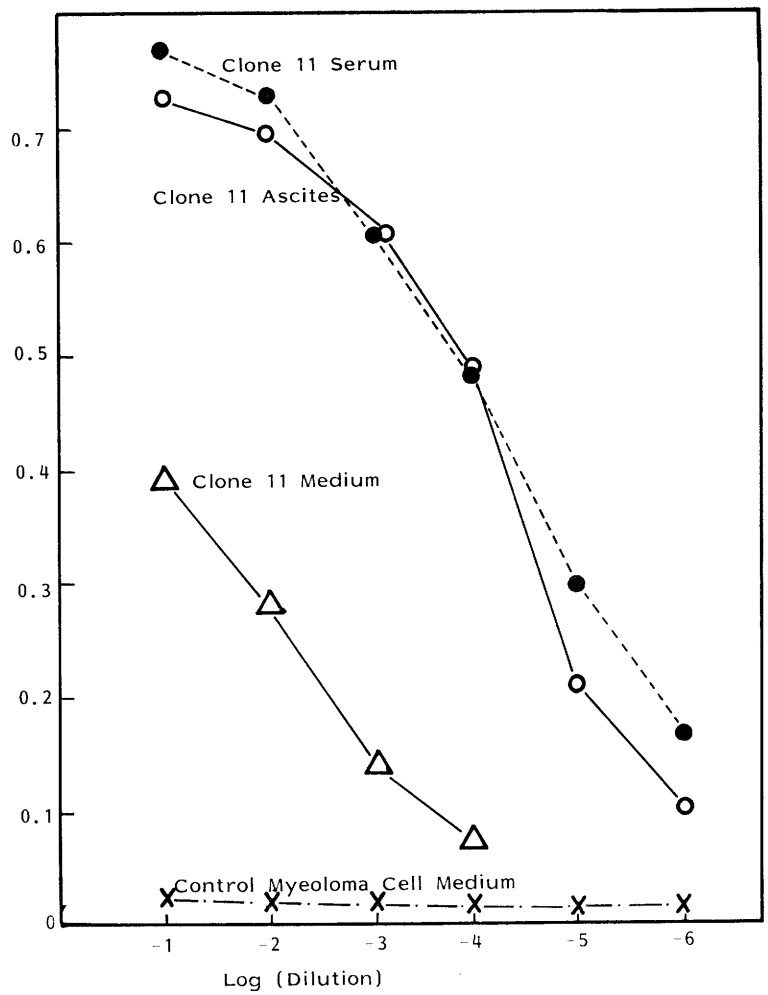


Fig. 1. ELISA test against Japanese encephalitis virus antigen with clone 11 culture medium and mouse ascites and serum.

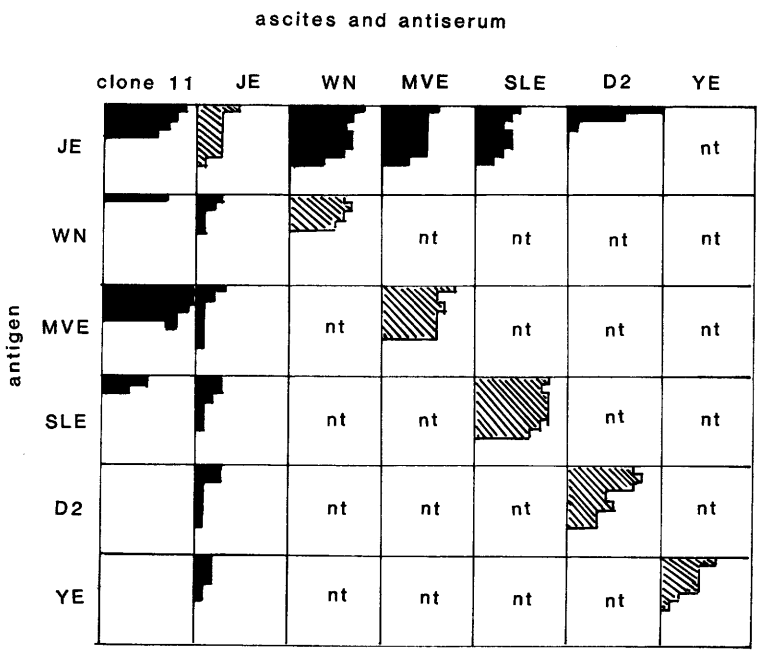
Characterization of hybridoma clones:

None of the clones gave positive results by the neutralization test applying the BHK21 cell culture. The hemagglutination inhibition test was used to detect the cross-reactivity between JE virus and various strains of arboviruses. All ascites fluids produced by the hybridoma clones revealed the cross-reaction among strains of Nakayama and JaGAr of JE virus, Malley Valley encephalitis virus and West Nile virus. However, the weak reaction against St. Louis encephalitis virus and missing reaction against yellow fever virus and dengue viruses were demonstrated in the ascites fluid and the serum. Since all hybridoma cell clones presented almost similar properties in the heagglutination inhibition test, it was ascribed eventually to derive from independent clones with similar specificity (Table 3). The characterization of ascites fluid produced by hybridoma cells

Table 3. HI test of mouse ascites against arbovirus antigens

Clones	Antigens								
	Nakayama	JaGAr	MVE	WN	SLE	YE	DEN-1	SIND	GET
1	6400	6400	12800	6400	800	<10	<10	<10	<10
4	12800	6400	6400	3200	400	<10	<10	<10	<10
5	6400	6400	6400	12800	200	<10	<10	<10	<10
11	3200	3200	6400	3200	100	<10	<10	<10	<10
14	3200	6400	6400	12800	400	<10	<10	<10	<10
20	3200	1600	6400	6400	100	<10	<10	<10	<10
Immun. mouse*	6400	1600	3200	6400	800	1600	80	<10	<10
Standard ant!-JE**	3200	3200	3200	3200	400	800	80	<10	<10

* Serum from immunized Balb/C mouse whose spleens were used for fusion experiment
** Standard hyperimmune anti-JE mouse serum



JE: Japanese encephalitis virus
WN: West Nile fever virus
MVE: Mulley Valley encephalitis virus
SLE: St Louis encephalitis virus
D2: Degue virus type 2
YE: Yellow fever virus
nt: not tested

Fig. 2. Cross reaction by the complement fixation test

in the complement fixation test indicated similar results of the hemagglutination inhibition test, however, it was noticeable that the antibody produced by the hybridoma cells reacted more remarkably against JE and Malley Valley encephalitis viruses than the other viruses (Figure 2).

Identification of immunoglobulin class by the immunodiffusion in agar:

The immunoglobulin class produced by the hybridoma clone cells were identified by the Ouchterlony immunodiffusion in agar as described in the materials and methods. As shown in the figure 3, the serum obtained from tumor bearing mice and the ascites fluid derived from the hybridoma cell clone 11 produced only one precipitation line against anti-mouse IgG3 (Figure 3).

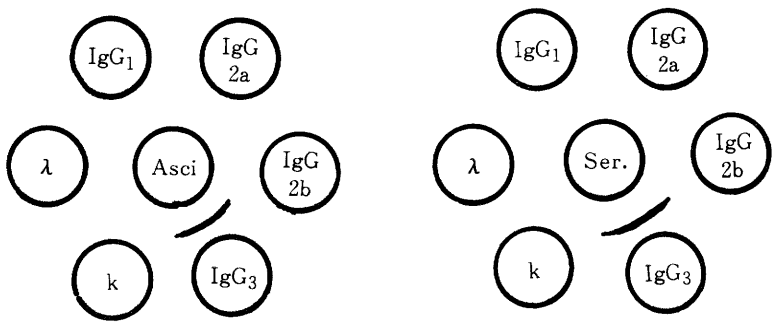


Fig. 3. Identification of immunoglobuline class by immunodiffusion in agar

Neutralization and challengege tests:

The antibody produced by the hybridoma cell clone 11 did not seem to have the significant neutralization activity which reacted with wild strain (B18 A) or the Nakayama strain of JE virus and West Nile virus in vitro or in vivo (Table 4, 5).

Table 4. *In vitro* Neutralization Test
(Virus dilution with constant ascites)

Virus	JE			WN
	Nakayama	B18A		
Ascites		Exp-1	Exp-2	
Control	4.8	4.8	4.6	5.5
Clone 11	4.6	4.1	3.5	5.8
Difference	0.2	0.7	1.1	−0.3

Remarks: Log (LD_{50}/0.01ml) in adult mice challenged intracerebrdally is calculated by Reed and Muench method.

Table 5. *In vivo* Challenge Test
(Ascites 0.01ml i. p. followed by i.c. challenge 6 hours later)

Virus / Ascites	JE	
	Nakayama	B18A
Control	6.0	5.3
Clone 11	5.4	4.3
Difference	0.6	1.0

Remarks: See Table 4.

DISCUSSION

It is known that the antibody produced by the hybridoma cells derived from the NS-1 myeloma cell contains the subclass of own immunoglobulin which may secrete either the definite light chain carried by the parent spleen cells or the k light chain secreted from NS-1 myeloma cell itself. In order to avoid the contamination of these subclass of immunoglobulin, the first experiment was performed by using the 63-Ag8/653 cell, because the cell strain does not contribute to secrete its own immunoglobulin subclass. However, the fusion frequency between the 63-Ag8/653 cell and the immune spleen cells was not high and the antibody production was not stable.

So far, from the fusion experiment between NS-1 myeloma cells and immune spleen cells, hybridoma cell clones produced the antibody against JE virus antigen. It was demonstrated that the antibody produced in this study exhibited the hemagglutination inhibition activity against the subgroup of the flaviviruses including JE, Malley Valley encephalitis and West Nile viruses, whereas the neutralization antibody related to the specific antigen was not clearly detected in the ascites fluid produced by the hybridoma cells derived from the NS-1 strain. As regard the antigenic specificity of viral components, Qureshi and Trent (1973) have indicated that the envelope glycoprotein of group B arboviruses contained both group and type specific determinants on the same molecule. Kitano, *et al.*, (1974) reported that the envelope of JE virus possessed hemagglutinating activity as well as neutralizing antibody blocking activity. However, the results of present study suggested that the monoclonal antibody produced by the hybridoma cells derived from the NS-1 strain was one of the antibodies against the subgroup specific determinant(s) of JE virus which is common to Malley Valley encephalitis, West Nile and St. Louis encephalitis viruses and the antigenic site(s) appear to have hemagglutinating but not neutralizing activities. Recently, Kimura, *et al.*, (1981) reported the antigenic analysis of JE virus with reference to the characterization of monoclonal antibody preparation. Furthermore, Kobayashi, *et al.*, (1982) repoeted the immunological

investigation on JE virus applying the monoclonal antibody. These studies must be noticed for the demonstration of antigenic determinant(s) of JE virus.

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モノクロナール抗体による日本脳炎ウイルスの抗原の解析

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最近，モノクロナール抗体は種々の抗原物質の分析に用いられ，その成果は著しい．本研究はモノクロナール抗体を用いて日本脳炎（日脳）ウイルスの抗原の解析を意企して行った．P3/63-Ag8/653，NS−1 および SP−2/0−Ag14 の3種の腫瘍細胞株を用い日脳ウイルスワクチン免疫 Balb/C マウス脾細胞との融和実験を行った．最終的には ELISA で日脳ウイルスに対する抗体を産生する hybridoma 細胞は NS−1 細胞由来のもので，それらをクローンとした9株が得られた．これらの hybridoma から得られたマウス腹水および抗血清には日脳，Malley Valley (MV)，West Nile (WN) の各ウイルスに対する血球凝集抑制抗体を含んでいたが中和抗体は証明出来なかった．今回は日脳ウイルスのみに特異的なモノクロナール抗体は得られなかったが，日脳ウイルスには MV ウイルス WN ウイルスおよび St Louis 脳炎ウイルスに共通の flavirus subgroup の特異抗原の存在が認められた．

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