Monoclonal Antibodies Specific for Dengue Virus Type 3

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Mouse lymphocyte hybridomas were prepared by polyethylene glycol-mediated fusion of cells from a mouse plasmacytoma line with lymphocytes from a mouse hyperimmunized with dengue virus type 3 (dengue-3). Media from 50 hybrid colonies were screened; 46 of them showed antibody activity against dengue-3-infected cells as determined by an indirect immunofluorescent antibody technique. Dengue monoclonal antibody obtained after cloning one of these colonies demonstrated activity in hemagglutination inhibition and indirect immunofluorescent antibody assays with dengue-3 antigen, but not type 1, 2, and 4 antigens. In addition, this antibody activity could be removed from culture media only by absorption with dengue-3 antigen.

Epidemics of infection with dengue virus, a mosquito-borne flavivirus, have been reported throughout the Caribbean area and northern South America in recent years (12). Such epidemics not only affect the health of a given population but can also be an economic burden (21). Control of these epidemics depends upon early identification and close epidemiological monitoring of the infecting virus, coordinated with mosquito control measures. There are four recognized serotypes of dengue virus; three of these types (1, 2, and 3) appear to be the primary cause of dengue infection in the western hemisphere, and type 4 has been associated with severe dengue infection in the eastern hemisphere, particularly in Southeast Asia. It is important to determine the antigenic type of dengue virus prevalent in a given area, because of the possibility of dengue virus vaccines (4, 16, 17) being used to prevent or control infections in epidemic or endemic areas, and for monitoring the spread of virus from country to country. This is difficult because the antisera currently used for dengue virus identification are highly cross-reactive between the four serotypes.

The method least subject to interference by dengue cross-reactive antibodies is plaque reduction neutralization (1, 15). However, it is time consuming and is not a feasible technique for typing a large number of isolates. The complement fixation technique is also used to identify dengue virus serotypes, but obtaining sufficient quantities of complement-fixing antigen from virus isolates is difficult and often requires passage of the virus in animals or cell culture (5, 13). Kuberski and Rosen used mosquitoes to produce high levels of complement-fixing antigen, but found most hyperimmune mouse ascites fluid antibody preparations used in their assays

to be excessively cross-reactive (11). An immunofluorescent-antibody (IFA) technique has worked well for early detection of viral antigen in infected mosquitoes, but is also highly cross-reactive (10).

The preparation of monospecific antisera or hyperimmune mouse ascites fluid to any of the four dengue serotypes is difficult because the viral antigen preparations commonly used for immunization (infected suckling mouse brain [SMB] or purified virions) contain both cross-reactive and type-specific antigens, resulting in antibody preparations that are heterogeneous. The use of purified viral protein for immunization might improve specificity, but a prime candidate for this, gp-58 (formerly VP-3), which is associated with viral neutralization and hemagglutination (8), also contains group- and complex-reactive determinants (20).

An alternative approach for producing monospecific antibodies that would not require purified type-specific antigen involves the production of monoclonal antibodies from mouse lymphocyte hybridomas (6, 9). This technique permits the antibody response of a mouse to be sorted into clones of hybrid cells that produce antibody directed against a single antigenic determinant. Using this technique, we have isolated 50 hybridoma cell colonies prepared from mice immunized with dengue virus type 3 (dengue-3)-infected SMB. At least one of these colonies and a clone derived from it secrete antibody that reacts type-specifically with dengue-3 viral antigen, and not with dengue types 1, 2, and 4.

MATERIALS AND METHODS

Viruses. Dengue types 1 (Hawaii), 2 (New Guinea C), and 4 (H241) were obtained from Robert Shope

(Yale University, New Haven, Conn.), and type 3 (PR-6) was obtained from P. Russell (Walter Reed Army Medical Center, Washington, D.C.). Virus stocks were passed in SMB (Swiss White mice) (2) and stored as 20% SMB suspensions in 0.05 M borate-buffered saline (pH 9.0) at -70°C .

Cell cultures. *Aedes albopictus* clone C6/36 was obtained from Akira Igarashi (Osaka University, Osaka, Japan) and grown in Eagle minimal essential medium (Flow Laboratories, McLean, Va.), containing 10% heat-inactivated fetal bovine serum, 0.2 mM non-essential amino acids (GIBCO Laboratories, Grand Island, N.Y.), 16 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 50 U of penicillin per ml, and 50 mg of streptomycin per ml. Cultures were maintained in a humidified, 28°C incubator.

Mouse plasmacytoma cells, Sp²/0-Ag14 (Sp cells), described by Shulman et al. (18), were obtained from Howard Gratzner (University of Miami, Miami, Fla.) and grown in D-MEM medium: Dulbecco-modified minimal essential medium with high glucose (4.5 g/liter) containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Cultures were maintained at 37°C with a humidified 5% CO_2 atmosphere.

Mouse lymphocyte hybridization technique. BALB/c mice were immunized by injecting 0.1 ml of Freund complete adjuvant intraperitoneally on day 1 and 0.4 ml of dengue-3 20% SMB subcutaneously and 0.1 ml intramuscularly on days 1 and 3. On day 30, 0.1 ml of Freund incomplete adjuvant and 0.2 ml of SMB were injected intraperitoneally. The mice were sacrificed 3 days later to obtain splenocytes for cell hybridization by a modification of the procedure described by Kennet et al. (7). Peritoneal macrophages, which are required as a feeder layer for the hybrids, were collected before the splenocytes by peritoneal lavage with 10 ml of D-MEM medium and washed by centrifugation at $500 \times g$ for 10 min. The pellet was suspended in 5 ml of D-MEM medium and stored at room temperature until needed.

Splenocytes were harvested from a single excised spleen by perfusing it with 10 ml of D-MEM medium containing 20% fetal bovine serum. The cells (approximately 10^6) were washed by centrifugation at $500 \times g$ for 10 min at room temperature and suspended in ice-cold, 0.17 M NH_4Cl to lyse the erythrocytes. The cells were immediately centrifuged at $500 \times g$ for 10 min at 4°C , suspended with 5 ml of D-MEM with 20% fetal bovine serum, pelleted again, and suspended in 5 ml of D-MEM without serum, containing 10^7 Sp cells. After pelleting once more, the cells were fused by suspending in 0.5 ml of D-MEM without serum and containing 30% polyethylene glycol (molecular weight, 1,000; Aldrich, Milwaukee, Wis.), incubated at room temperature for 2 min, and then centrifuged for 6 min at $500 \times g$. The pellet was gently suspended with 5 ml of D-MEM without serum, centrifuged at $500 \times g$ for 10 min, and suspended with the peritoneal macrophages in 40 ml of HY medium (D-MEM without serum, with 10% NCTC 109 [Microbiological Associates, Walkersville, Md.], 0.2 U of bovine insulin per ml, 0.45 mM pyruvate, 1.6×10^{-5} M thymidine, and 10^{-4} M hypoxanthine). The cells were distributed into

four 96-well microtiter plates using 1 drop per well (approximately 10^6 cells).

After incubation at 37°C for 24 h, 1 drop of HY medium containing 20 μg of methotrexate per ml was added to each well. On days 3 and 5 one drop of HY medium containing 10 μg of methotrexate per ml (HAT medium) was added. Colonies became visible in 7 to 10 days in approximately 30% of the wells, at which time the medium was removed and replaced with 0.25 ml of HAT medium. When these media became acidic they were removed and screened for antibody activity.

Antibody screening. Uninfected C6/36 cells and C6/36 cells persistently infected with dengue-3 were grown in eight-chamber tissue culture slides (Lab-Tek, Naperville, Ill.) until confluent. The chambers were then washed twice with phosphate-buffered saline (pH 7.4), fixed in -20°C methanol for 15 min, and washed once with phosphate-buffered saline; then 0.1 ml of medium from a well containing a colony of hybrid cells was added to a chamber of infected and uninfected cells. Wells that contained multiple colonies of cells were not tested. After incubation for 45 min at room temperature, the chambers were washed three times in phosphate-buffered saline, 0.1 ml of fluorescein-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Miles Laboratories) was added, and slides were then incubated for 45 min at room temperature. After washing three times with phosphate-buffered saline, the chambers were removed, and the slide was flooded with phosphate-buffered saline and immediately examined using a Leitz Ortholux fluorescent microscope with an HBO 200-W mercury lamp and BG-38 heat-absorbing, BG-12 exciter, and K-510 suppression filters.

Antibody production. Hybrid cells from wells containing the desired antibody activity were transferred to 24-well tissue culture plates (Flow Laboratories) containing 1 ml of HAT medium and 10^6 peritoneal macrophages per well. When a density of 3×10^5 to 5×10^5 hybrid cells per well was reached, the cells were transferred to culture tubes (15 by 125 mm) with 10 ml of HAT medium. At this point, hybrid cells selected for continued passage were cloned using a soft-agar technique (7). Conditioned media for use in subsequent studies were pooled from cultures of each clone after the medium became acidic (approximately 10^6 cells per ml). Antibody was also obtained from mouse ascites fluid by passage of the hybrid cells in pristane-primed BALB/c mice (7).

HI assay. Hemagglutination inhibition (HI) assays were done by a microtiter modification of the method reported by Clark and Casals (2). Hemagglutination antigen was prepared by a sucrose-acetone extraction of infected SMB (8). It was not necessary to treat media from the hybrid clones to remove nonspecific hemagglutinating activity. Media from a hybrid clone producing antibody to tumor antigen material was used as a negative control.

IFA. Media samples were serially diluted in HY medium, and 0.1 ml was added to dengue-infected C6/36 cells in tissue culture chambers. The cells were stained and examined as described above. The antibody titer was reported as the reciprocal of the last dilution of medium giving fluorescence distinguishable

from tests with uninfected control cells.

Antibody absorption. Media containing antibody were absorbed with dengue antigen or a control material prepared from normal SMB by mixing 20 μ l of medium with 500 hemagglutination units of dengue hemagglutination antigen and incubating at 37°C for 1 h. The remaining antibody activity was assayed by IFA technique.

Characterization of immunoglobulins. Media samples from the clones were concentrated 10-fold before testing, using an immersible CX filtering system (Millipore Corp., Bedford, Mass.). Immunodiffusion tests were performed using an immunodiffusion kit (Miles Laboratories, Elkhart, Ind.) with 2% agarose slides. Immunoelectrophoresis was done according to the manufacturer's specifications on a Gelman deluxe electrophoresis apparatus. Goat anti-mouse immunoglobulin (IgG, IgM, and IgA, heavy and light chain) was obtained from Cappel Laboratories, Cochranville, Pa.

RESULTS

Screening of hybrid clones. Media samples from 50 microtiter wells containing individual colonies were screened for antibody directed against dengue-3 antigen. Indirect immunofluorescent staining was observed with 46 of the samples. Media from 12 of these 46 colonies were further tested for binding to dengue-1-infected cells, and three, wells 31, 33, and 36, did not bind. Cells from two of these wells, 31 and 36, and two wells containing antibody that did bind to dengue-1-infected cells, 20 and 27, were cloned and used in subsequent studies.

Antibody immunoglobulin types. Concentrated media samples from all four clones were tested by immunoelectrophoresis and demonstrated a single precipitin band against goat anti-mouse immunoglobulin. The electrophoretic mobility of the band was similar to that of mouse IgG. Using double immunodiffusion, one precipitin band with identity between media and normal mouse serum was observed when run against goat anti-mouse IgG.

HI titer. All four clones produced antibody with HI activity against dengue-3 antigen (Table 1). Antibody from two of the clones, 31 and 36, did not demonstrate activity with antigen from the other three serotypes. Antibody from clones 20 and 27 were cross-reactive, with each having similar titers with all four serotypes. Mouse ascites fluid obtained by using clone 31 contained the highest HI titer tested against dengue-3 and demonstrated negligible cross-reactive antibody activity.

Immunofluorescent staining and antibody titer. Antibodies in the media from the clones were observed to produce extremely low levels of nonspecific, background staining of uninfected cells, even when undiluted culture medium was used. This was in contrast to our

experience with staining with hyperimmune mouse ascites fluid, which had to be repeatedly absorbed with uninfected cells to reduce nonspecific staining. The staining pattern observed with the culture media appeared mainly as large cytoplasmic inclusions and scattered granules.

The IFA titers to dengue-3-infected cells of antibody from all four clones were within one twofold dilution (Table 2). Antibody from clone 20 reacted equally well with cells infected with each of the four serotypes. Clone 27 antibody had four- to eightfold lower titers against dengue-1, -2, and -4 than against type 3. Antibody from clones 31 and 36 stained only dengue-3-infected cells throughout the range of dilutions used, even when the media were concentrated 10-fold. This type specificity of antibody from clone 31 was maintained in mouse ascites fluid, which demonstrated the highest titer of all samples tested against dengue-3 and did not stain cells infected with the other three types.

Absorption of antibodies. Media from clones 20 and 31 were absorbed with hemagglutinating antigen from each of the four dengue serotypes and uninfected SMB and then assayed for antibody activity against dengue-3-infected cells by IFA (Table 3). Antibody activity from clone 31 medium was removed by absorption with dengue-3 antigen but not by any of the other antigens. Clone 20 antibody activity was removed by absorption with all of the dengue antigens.

DISCUSSION

Several mouse lymphocyte hybridoma clones which produce antibody that binds to dengue antigen were isolated. In these experiments, antibody from at least one of these clones (clone 31) was found to be specific for dengue-3 virus

TABLE 1. Comparison of reciprocal HI titers of anti-dengue-3 monoclonal antibodies against four dengue virus serotypes

Medium from clone no.	Reciprocal HI titer with antigen:			
	Dengue-1	Dengue-2	Dengue-3	Dengue-4
20	16	32	32	16
27	16	8	16	8
31	4	4	32	4
36	4	8	64	4
Control ^a	4	4	4	4
31 ^b	16	32	1,024	16
Control ^c	8	8	16	8

^a Medium from a clone producing antibody to tumor antigen.

^b Mouse ascites fluid.

^c Mouse ascites fluid obtained by using a clone not secreting immunoglobulin.

TABLE 2. *Comparison of reciprocal IFA titers of anti-dengue-3 monoclonal antibodies against dengue virus-infected cells*

Medium from clone no.	Reciprocal IFA titer			
	Dengue-1	Dengue-2	Dengue-3	Dengue-4
20	64	32	64	32
27	8	4	32	4
31	0	0	32	0
36	0	0	64	0
31 ^a	0	0	256	0
36 ^a	0	0	256	0
31 ^b	0	0	2,048	0

^a Medium concentrated 10-fold.^b Mouse ascites fluid.TABLE 3. *Effect of absorption of anti-dengue-3 monoclonal antibodies with dengue antigens upon the reciprocal HI titer against dengue-3*

Medium from clone no.	Reciprocal HI titer after absorption with antigen:				
	Control ^a	Dengue-1	Dengue-2	Dengue-3	Dengue-4
20	64	0	0	0	0
31	64	64	64	0	64

^a Antigen prepared from uninfected SMB.

when assayed by the HI and IFA techniques. Proof of the specific nature of this antibody was obtained when absorption with dengue-3 antigen removed the IFA activity for dengue-3-infected cells, whereas absorption with dengue types 1, 2, and 4 did not. Conversely, clone 20 was highly cross-reactive and demonstrated equivalent antibody activity with all dengue serotypes tested. The immunoglobulin secreted by all four clones was of the IgG class.

From our data, it was not possible to accurately determine which viral protein was responsible for production of the type-specific monoclonal antibody. Since all four clones tested exhibited HI activity, it is possible that the monoclonal antibody activity from clone 31 was directed to a type-specific determinant on the envelope glycoprotein, gp-58 (20), which is involved in the viral hemagglutination reaction (8). This glycoprotein has also been shown to possess group- and complex-reactive determinants (20), and hybridomas prepared against this material could be expected to be either type specific (clone 31) or cross-reactive, such as antibody from clone 20. In addition, the probability of obtaining a hybridoma to gp-58 was relatively high, since it is the most abundant viral structural protein and a majority of the animal's antibody response might be directed to it. Final determination of the specific dengue-3 antigens

responsible for the production of each monoclonal antibody will require study of the antibody with purified viral protein.

An important potential application of dengue type-specific monoclonal antibodies is in type-specific identification of dengue virus isolated during epidemics. Currently, routine identification procedures require at least 2 weeks, using methods that first isolate the virus in mosquitoes or cell culture, followed by a complement fixation assay of the infected tissue (10, 22). Recently, C6/36 cells were used in a similar manner to isolate and identify dengue viruses in 6 days (19). It is likely that the time and effort required for identification could be significantly reduced if the virus could be specifically typed directly in inoculated cell cultures or mosquitoes. Viral antigens can be detected in mosquitoes infected with patient sera by direct immunofluorescence in 6 to 10 days (10, 19, 22). Similar techniques might provide type-specific identifications if a panel of dengue or other arbovirus monoclonal antibodies were available. It is conceivable that monoclonal antibodies might also be used to classify viral isolates within a serotype. Dengue-3 has been reported to consist of two subtypes (14). The antibody from clone 31 was only tested against the PR-6 strain of dengue-3, but is currently being tested against other dengue-3 strains for subtype specificity.

Dengue monoclonal antibodies might also be used to obtain highly purified viral proteins by affinity chromatography. Such purified material could be used for further characterization of viral proteins and antigen structure, which could aid in understanding complications involved with secondary dengue infections (16). If an antigen could be found that contains only type-specific determinants, it could be used in a method such as an indirect enzyme-linked immunosorbent assay (3) to detect type-specific antibodies in patients' sera.

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