Epstein-Barr Virus Viral Capsid Antigen Titer by Immunofluorescence with Microplates: New Semiautomated Method Based on the Microtiter System

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Received for publication 24 February 1977

A semiautomated method of an indirect immunofluorescence technique for the titration of antibodies directed against viral capsid antigens of Epstein-Barr virus has been developed with Microtiter system units. By this method, a technician is able to titrate some hundred samples daily. The technique is safe, easy, and reproducible. The various procedures are described, and the sensitivity of the test is discussed.

Henle and Henle established the relationship between Epstein-Barr virus (EBV) and mononucleosis syndrome (3). Thereafter, titration of antibodies directed against a viral capsid antigen (VCA) of this virus by immunofluorescence has enabled workers to detect nonimmunized subjects, to study the epidemiology of the virus, and to establish the diagnosis of mononucleosis, mainly in children as well as in adults who show no change with Paul and Bunnell's test. The EBV-VCA antibodies are routinely titrated on Burkitt P3J-HR1K (5) cell smears by an indirect immunofluorescence method. The technique originally described used smears on glass slides (2). In our laboratory, the titration of EBV-VCA antibodies by immunofluorescence was performed for 6 years on smears obtained by cytocentrifugation on glass slides. The method is highly reproducible, but it needs numerous and tedious manipulations while carrying a risk of contamination, for instance in the case of hepatitis B antigen-positive sera. For these reasons, we considered the elaboration of a new method useful. This new technique is based on the Microtiter system (Cooke Engineering Co., Alexandria, Va.). Readings of the revised test are effected directly on the bottom of microplate wells by means of an inverted fluorescence microscope. This method appears to be reliable and much more efficient. We describe the method and discuss the sensitivity of the test.

MATERIALS AND METHODS

Cell culture. In an example typical of the test's performance, the P3J-HR1K strain, kindly provided by G. E. Moore, was grown in the dark at 37°C in a 4-liter round-bottom flask with continuous agitation. Cell concentration was adjusted each day to 0.5×10^6 cells/ml by a supply of fresh medium.

Medium and buffer. The medium used was RPMI 1640 with 10% calf serum (decomplemented, 30 min at 56°C), 2 mM glutamine, 100 U of penicillin per ml, and 50 μ g of streptomycin per ml. The solution used for washing the cells in phosphate-buffered saline (PBS) was composed as follows (in grams per liter): KH_2PO_4 , 0.45; Na_2HPO_4 , 3.44; and NaCl, 7.2 (pH 7.2).

Reagents for fluorescence. Sera from patients were decomplemented (30 min at 56°C). The buffer used was composed of four parts of a solution A (9 g of NaCl and 1.42 g of Na_2HPO_4 per liter) and one part of a solution B (9 g of NaCl and 1.38 g of Na_2HPO_4 per liter), mixed before use (pH 7.2).

Culture of cells for the immunofluorescence test. Before being used as source of antigen for the immunofluorescence test, P3J-HR1K cells, from the stock culture, were centrifuged and maintained in stationary culture in Roux flasks at a concentration of 0.3×10^6 cells/ml, 200 ml per flask, for 3 days at 33°C without addition of medium.

Preparation of microplates. After 3 days at 33°C, the cells were centrifuged in a Christ IKS centrifuge at room temperature at 1,500 rpm ($500 \times g$) for 10 min; they were then washed three times in PBS and suspended in PBS at the concentration of 0.8×10^6 cells/ml. The suspension of cells was distributed over the Falcon Microtest II (flat bottom) microplates, 0.1 ml/well, with an automatic Cornwall pipette. These microplates were centrifuged in the Christ IKS centrifuge, fitted with microplate rotor, at 250 rpm (i.e., $15 \times g$) for 10 min. The microplates were then turned upside down and left to dry for at least 6 h at room temperature. Next, the cells were fixed during 10 min with lighter fluid, a mixture containing: 0.15% benzene, 1.33% toluene, 0.69% ethylbenzene, 4.66% xylene, and 93.17% paraffinic hydrocarbons (U.S.A. Petroleum Chemicals). The microplates were again turned upside down and allowed to dry from 15 to 24 h at room temperature, and afterwards, wrapped in aluminum foil, they were stored at -20°C until required for use. Before use, each batch of prepared microplates was tested with the reference serum.

Indirect immunofluorescence test. For each serum, a twofold serial dilution was made in the 12 wells of a row of a vinyl transfer plate (Cooke). The first well of the dilution sequence received the 1:4 dilution of patient sera. Vinyl transfer plates were used with a plate holder for this purpose. The Falcon microplates, the flat bottom wells of which had previously been lined with cells as described above, were thawed, and the cells were rehydrated with 0.1 ml of phosphate buffer. After 10 min, the buffer was eliminated by turning the microplates upside down, and the sera were transferred to the cells by capillarity by means of a system of transfer plates (Fig. 1). In each microplate, one row of wells served as control cells. After 30 min of incubation in a moist chamber at 37°C, the microplates were turned upside down and washed three times with the buffer. To each well was added one drop of a 1:60 dilution of goat antihuman immunoglobulin G conjugated with fluorescein isothiocyanate (Hyland Laboratories, Inc., Costa Mesa, Calif.) and mixed with a 1:300 dilution of a counterstain consisting of bovine albumin labeled with rhodamine (Baltimore Biological Laboratories, Cockeysville, Md.). After a further incubation for 30 min at 37°C in a moist chamber, the plates were again turned upside down and washed three times with the buffer. One drop of buffer was then placed in each well. Reading was effected by means of an inverted fluorescence microscope, Diavert (Leitz/Opto-Metric Div. of Leitz Inc., Rockleigh, N.J.), Ploem Opak 2, equipped with a HBO-50 W mercury lamp and a special stage for microplates (Leitz). This reading can be taken immediately, or it may be delayed up to several days, provided the microplates are kept at 4°C in a moist chamber and in darkness. The reading was taken row by row, i.e., serum by serum, beginning with the first dilution. The whole of each well was examined. The titer corresponded to the last dilution where fluorescent cells with the characteristic

aspect of EBV-VCA antigen were detected (Fig. 2). The result is expressed by the base 2 logarithm of the reciprocal of this dilution.

RESULTS

Optimum conditions for production of EBV-VCA. The culture of P3J-HR1K cells in a 4-liter round-bottom flask with continuous agitation enables large quantities of cells to be produced. The viability of the cells tested by exclusion with trypan blue is from 97 to 100%. The percentage of mitoses (May-Grünwald-Giemsa staining) is 2%. The doubling time of a cell population is about 30 h. Nevertheless, before using these cells for the immunofluorescence test, we have chosen to keep them in stationary subculture at 33°C without addition of medium. Several authors before us have observed an increase of the EBV-VCA production by the P3J-HR1K strain in such conditions (1, 4, 6). The starting concentration of subcultures was fixed at 0.3×10^6 cells/ml because it ensured better survival of the cells without addition of medium. To determine the optimum time of the subculture at 33°C, we tested the P3J-HR1K cells while kept at that temperature for 5 days. Each day (i.e., on days 0, 1, 2, 3, 4, and 5) cells were prepared on microplates by the above described procedure and were then used for titrating the reference serum (Fig. 3). The viability of the cells measured by the trypan blue exclusion test was higher than 90% throughout the testing period. On the basis of those results, it was decided that the cultures would be kept stationary for 4 days before the preparations of microplates. After testing con-

FIG. 1. Picture shows the serum drop passing from plastic transfer plate to bottom of microplate well by capillary attraction.

FIG. 2. Picture of P3J-HR1K smears in the microplate was taken through Leitz inverted microscope: positive cells show brilliant VCA immunofluorescence pattern ($\times 250$).

FIG. 3. Influence of duration of subculture on titer of a reference serum. Cells fed with 1% calf serum (●) and cells fed with 10% calf serum (○) were used to estimate the titer of a reference serum. Each point represents the mean of 30 assays.

centrations varying from 0.4×10^5 to 1.2×10^5 cells/well, the optimum concentration, in view of obtaining a homogenous layer with a minimum of superposition, appeared to be between 0.6×10^5 and 0.8×10^5 cells/well. Centrifugation speed of the microplates is critical for ob-

taining a homogeneous lining. We use 250 rpm for 10 min at room temperature, in the Christ IKS centrifuge, with microplate rotor ($15 \times g$). Above that speed the cells are deposited as a ring around the periphery of the well bottom, and such superposition makes any accurate reading impossible. It was necessary to work out a method for fixing the cells at the flat bottom of the plastic microplates. Preliminary trials with different fixing agents were performed with centrifuged smears on glass slides. The same antigen corresponding to a preparation of P3J-HR1K cells was fixed with various products and titrated with the same reference serum. We obtained the highest titers ($12 \log_2$) with the cells fixed with acetone, carbon tetrachloride, and lighter fluid. The other fixing agents gave a lower titer, varying from $11 \log_2$ for the ethanol-acetone mixture (70:30, vol/vol) to $2 \log_2$ for the ethanol-ether mixture (70:30, vol/vol). For the purpose of fixing smears on microplates, acetone and carbon tetrachloride were discarded, since they react with the material of the microplates. The other fixing agents we have tested (methanol and ethanol) gave lower titers (Table 1). Thus, we came to choose lighter fluid as the fixing agent: the lymphoblastoid cells fixed by lighter fluid retain their usual morphology when examined by May-

TABLE 1. Modes of fixation

Mode	Fixation liquid	Temp	Titer of reference serum (EBV-VCA) ($\log_2$)
Glass slides	Acetone	Room	12
	Carbon tetrachloride	Room	12
	Lighter fluid	Room	12
	Ethanol-acetone 70:30 (vol/vol)	Room	11
	Isopentane	-20°C	9
	Ethanol	Room	8
	Methanol	Room	8
	Ethanol-acetone 90:10 (vol/vol)	Room	8
	Ethanol-acetone 80:20 (vol/vol)	Room	8
	Ethanol-ether 90:10 (vol/vol)	Room	6
	Ethanol-ether 80:20 (vol/vol)	Room	6
	Ethanol-ether 70:30 (vol/vol)	Room	2
Microplates	Lighter fluid	Room	12
	Ethanol	Room	8
	Methanol	Room	7

Grünwald-Giemsa staining. The cell layer remains homogeneous during the various testing procedures; the immunofluorescence pattern and the antibody titer of the control serum are the same as those obtained on glass slides with acetone or carbon tetrachloride fixation. Comparative tests were effected on one and the same antigen batch after storing the microplates at -20°C for several months. No difference in antibody titer was seen with the reference serum after 1, 3, and 6 months.

Comparison of results obtained on slides and on microplates. Two hundred and seventeen serums previously tested on glass slides have been titrated again by our microplates system. Seventy-six serums (35.85%) displayed the same titer. For 84 serums, (39.62%) the titer on microplates differs from the result on slides by one dilution. Fifty-two serums (24.52%) show a difference of two or three dilutions between the titers. The results on microplates were generally somewhat lower than those obtained on glass slides. The correlation coefficient between the results obtained on glass slides and the results obtained on microplates is 0.9178, and the regression line was found to

have a y-intercept at 0.6210 and a coefficient of 0.8486 (Fig. 4).

Reproducibility of the method. We have investigated the constancy of the reference serum titer. The results of those experiments are summarized in Table 2. In each experiment, the reference serum is tested 30 times on one and the same antigen distributed among a series of microplates. The mean titer and its standard deviation are calculated for the reference serum. In the first series of experiments, the cells used as antigen were grown with 1% calf serum. In the second series, the cells were fed with 10% calf serum. The coefficient of variation for the 300 tests taken as a whole was found to be 0.07. We also have made a trial in order to estimate the general reproducibility of the titers that we obtain. We compared the results of 500 sera that have been tested twice. The sera were those tested twice in clinical diagnostic routine. The assays have been done at different days and on different batches of antigen. For 282 serums (56.40%), we have obtained the same result. For 194 (38.8%), there was a difference of one dilution. For 24 (4.8%), a two-dilution difference could be noted. The

FIG. 4. Comparison of titer of EBV-VCA antibodies: 217 serums have been tested on smears on glass slides (x-axis) and on microplates (y-axis).

correlation between the first and the second assay was 0.9634. For the 500 sera, we computed a regression line having a y-intercept at 0.3160 and a coefficient of 0.9596 (Fig. 5).

DISCUSSION

Titration of EBV-VCA antibodies by indirect immunofluorescence on P3J-HR1K in microplates is simple, useful, and highly reproducible. To obtain good results by this method, several technical procedures appear to be very

important: stock culture of the P3J-HR1K strain should be prepared in a 4-liter flask with continuous agitation, so that cell viability is maintained in the range of 97 to 99%. Stationary subcultures containing 0.3×10^6 cells/ml should be maintained at 33°C for 4 days without added medium. This procedure should give optimum synthesis of EBV-VCA corresponding in maximum sensitivity to that shown in the tests with the reference serum (Table 2). It should be noted that the proportion of calf se-

TABLE 2. Sensitivity of indirect immunofluorescence antibody test for EBV-VCA in microplates

Days at 33°C	Reference serum EBV-VCA titer					
	1% calf serum			10% calf serum		
	$\bar{X}^a$	$\sigma/\sqrt{n}$	$\sigma/\bar{X}$	$\bar{X}^a$	$\sigma/\sqrt{n}$	$\sigma/\bar{X}$
0	6	0.0840	0.0767	6	0.0840	0.0767
1	6.46	0.1077	0.1207	6.63	0.0849	0.0739
2	8.1	0.1057	0.0753	7.96	0.0988	0.0716
3	8.06	0.0832	0.596	8.11	0.1005	0.0715
4	9.9	0.0832	0.0485	9.7	0.1733	0.0619
5	9.05	0.1195	0.0762	8.47	0.0884	0.0602

^a $\bar{X}$, Mean of 30 titers of reference serum.

FIG. 5. Comparison between two titers obtained on microplates on different days from different antigen batches with the same serum. Five hundred serums have been tested twice.

rum (1 or 10%) in the subculture medium does not, to a significant extent, alter the viability of the cells, which remained higher than 90% after 5 days. The reference serum mean titer seemed somewhat higher on an antigen prepared with 1% serum; this difference, however, is not significant. In preparing the microplates, the most important factors are: the optical quality of the plastic component of the microplate, washing of the cell pellet with PBS, number of cells per well (0.8×10^5), speed at which microplates are centrifuged ($15 \times g$), prolonged drying of the cells before (6 h) and after (15 h) being fixed with lighter fluid, and storage at -20°C . We consider the method to present two main advantages: firstly, its very good yield in practice; one person can titer 120 sera a day with 12 dilutions for each serum, whereas by the glass-slide smear method this same person could not titer more than 10 sera a day with 7 dilutions for each serum; secondly, the method is safer; the manipulations demanded by the test are easy to carry out without risk of contamination by an infectious serum. This semiautomated method, based on the Microtiter system (Cooke) with transfer plate and immediate reading of the immunofluorescence at the

level of the microplate wells, will certainly lend itself to wide application for other tests of antibody in virology.

ACKNOWLEDGMENTS

We wish to thank J. Tondreau, B. Michot, and B. Pire for skillful technical assistance.

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