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Radioimmunoassay for Detection of Antibodies to Epstein-Barr Virus in Human Infectious Mononucleosis Serum Specimens

HARRIET D. HUTCHINSON,* DONALD W. ZIEGLER, AND PAUL M. FEORINO
Center for Disease Control, Public Health Service, Atlanta, Georgia 30333

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A rapid microradioimmunoassay (RIA) technique was adapted for quantitatively measuring antibody titers to antigens occurring in Epstein-Barr virus (EBV)-infected lymphoid cells. In these experiments two EBV-infected cell lines, HR1K and EB-3, were used as antigen-positive cells and Molt-4 was used as the negative control cells. The antibody titers of sera from suspected infectious mononucleosis patients were compared by RIA and indirect fluorescent antibody (IFA) methods. As determined by each of the methods, 14 of 19 sera had positive antibody titers and the remainder of the sera had negative antibody titers. Thus, the two methods agreed completely in differentiating sera with antibodies to EBV antigens. To further evaluate the antibody specificity of the RIA, the antibody titers of paired sera, pre- or early infection and postinfection, from five confirmed infectious mononucleosis patients were determined by RIA and IFA. Seroconversion was demonstrated by both RIA and IFA for each of the patients. Thus, the sensitivity and specificity of the two procedures are about the same.

The conventional assay used for detecting antibodies to Epstein-Barr viral (EBV) antigens is the indirect fluorescent antibody (IFA) assay described by Henle and Henle (4). Radioimmunoassay (RIA) methods for membrane antigen associated with EBV have been described (6, 11). In the procedures, multiple centrifugations of the samples are required; thus, these methods are not readily adaptable for use in diagnostic laboratories. We have successfully devised an alternate RIA method to measure antibodies in human sera to those antigens occurring in Epstein-Barr-infected lymphoid cells. The procedure is rapid and easy to perform and because the antigen is affixed to the wells of microtiter plates, numerous serum specimens can be processed simultaneously. The antigen in the RIA procedure is a crude cell homogenate similar to the antigen in IFA, hence the RIA probably measures viral capsid antigen and other antigens occurring in the infected lymphoid cells. The proposed indirect RIA method conceptually resembles the IFA method; therefore, the results of the two methods would be expected to be comparable.

In both IFA and RIA the nonspecific adsorption of the indicator globulin must be excluded. Nonspecific background fluorescence occurring in the IFA analytic system is reduced with optical filters, therefore only an EBV-infected cell line is needed. Contrariwise, in the RIA procedure both EBV-infected (antigen) and uninfected (control) transformed lymphoid cell lines are used. Nonspecific background adsorption of radioactivity is accounted for by subtracting the radioactivity adsorbed by an uninfected cell line. The proposed RIA method was adapted from methods developed to measure antibodies against smallpox and vaccinia viruses (9, 16).

MATERIALS AND METHODS

Cell lines. In this study several transformed lymphoid cell lines were used as the source of either antigen or control material. These included HR1K, EB-3, and Raji obtained from W. Henle (Children's Hospital, Philadelphia, Pa.), Molt-4 obtained from Roswell Park Memorial Institute (Buffalo, N.Y.), and NC-37 obtained from the Reagents Section, National Cancer Institute (Bethesda, Md.). The chronically EBV-infected transformed lymphoid cell lines, HR1K, and EB-3, were originally obtained from Burkitt lymphoma patients (1, 15). These cell lines have been reported to contain virus particles (7, 8), membrane antigen (13), early antigen (5), and viral genomic material (3, 15).

Each of three uninfected cell lines, NC-37, Raji, and Molt-4, was evaluated for use as negative controls in the RIA procedure. Raji cells were originally obtained from a Burkitt lymphoma patient (15), however NC-37 and Molt-4 are non-Burkitt-derived lymphoid cell lines (14, 15). Raji and NC-37 cells usually do not contain EBV capsid antigen (3) but have been shown to possess genetic information homologous with EBV-deoxyribonucleic acid (3, 15). Therefore, these two cell lines may not be ideal control cells for RIA. Molt-4 cells contain neither EBV antigens nor genetic information homologous with

EBV-deoxyribonucleic acid (12) and are probably a more suitable control cell line for RIA.

Antigen preparation. In preparing EBV-infected and control material, the cell suspensions were washed twice with phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of 4.0×10^6 to 5.0×10^6 cells/ml. The cell suspensions were sonically treated for 2 min in a 10 kc Raytheon sonic oscillator, and 0.05-ml volumes were added to siliconized polyvinyl flat bottom microtiter wells (Cooke Engineering Co., Alexandria, Va.) and air dried. The plates were stored unfixed at −20 C in desiccators containing a nitrogen atmosphere. Immediately before use both antigen and control cell materials in the microtiter plates were fixed with 10% buffered formalin, pH 7.0, for 15 min at 4 C and washed once with PBS. Uninfected cells fixed with formalin showed the lowest nonspecific adsorption of the radioactivity while the antigenic integrity of the infected cells was retained (16).

RIA procedure. Twofold serial dilutions of human sera were prepared in diluent consisting of 10% fetal calf serum in PBS. Each serum dilution was added in a volume of 0.025 ml, to duplicate wells containing EBV-infected cell material and to duplicate wells containing uninfected cell material. Each plate was covered and incubated at 37 C for 1 h. After incubation the serum dilutions were drained from the wells, and each plate was washed five times with PBS.

The lot of ^{125}I-labeled guinea pig anti-human globulin used in this study was diluted to contain 0.65 μg of protein per 0.025 ml in PBS containing 1% fetal calf serum. This dilution of ^{125}I-labeled anti-human globulin was the optimal concentration for maximal differentiation of sera of different antibody potencies (10). Aliquots (0.025 ml) were added to each of the microtiter wells, and each plate was again covered and incubated at 37 C for 1 h. After incubation the excess labeled globulin was drained and the plates were washed five times with PBS and dehydrated with 95% ethanol. Each microtiter well was clipped from the microtiter plate into plastic tubes (16 by 125 mm). The radioactivity in each microtiter well was counted in a gamma scintillation spectrometer.

The ^{125}I-labeled anti-human globulin specifically adsorbed by antibodies to EBV antigens was obtained by subtracting the radioactivity (counts per minute) adsorbed by the antiserum-control reaction from the radioactivity (counts per minute) adsorbed by the antiserum-antigen reaction. The serum titration curves were obtained by plotting the logarithm of the specifically adsorbed radioactivity on the ordinate and the logarithm of the reciprocal antiserum dilution on the abscissa. Serum end-point titers were dilution factors defined as the reciprocal antiserum dilution which adsorbed twice the radioactivity (counts per minute) adsorbed by the background controls (diluent). Antisera uniformly produced linear titration curves when plotted by this method; thus, graphic interpolation or extrapolation permitted determination of definitive end points. The validity of graphic determination of RIA end-point titers was confirmed by Student's *t* test. End-point titers determined by the two procedures were nearly the same. However, the coefficient of variation was much lower when graphic interpolation or extrapolation was used to determine the end-point titers.

IFA procedure. The EBV-IFA procedure was a modification (2) of the indirect fluorescent antibody test described by Henle and Henle (4). Suspensions of the chronically EBV-infected HR1K cell line were used as antigen in the IFA procedure.

RESULTS AND DISCUSSION

Initially the RIA procedure was developed using HR1K and NC-37 cell lines as the EBV-infected and uninfected antigen preparations, respectively. Typical RIA titration curves for four representative sera are shown in Fig. 1. The figure shows a negative serum (A) which is clearly distinguishable from the positive sera (B, C, and D). The titration curves of the three positive sera (B, C, and D) were linear over an eightfold to 16-fold range and yielded end-point titers of 125, 190, and 500, respectively.

The RIA results for 19 human diagnostic specimens (Table 1) were compared with those obtained by IFA. In this study HR1K cell material was the EBV antigen for both the RIA and the IFA procedures, and NC-37 cell material was the uninfected control for the RIA procedure. Five of the sera were negative by both methods and the remaining 14 sera had positive antibody titers by both methods. Thus, the two methods differentiated between sera with positive and negative titers. Of the positive sera, the titers of five were lower by RIA, the titers of three were equal by both methods, and

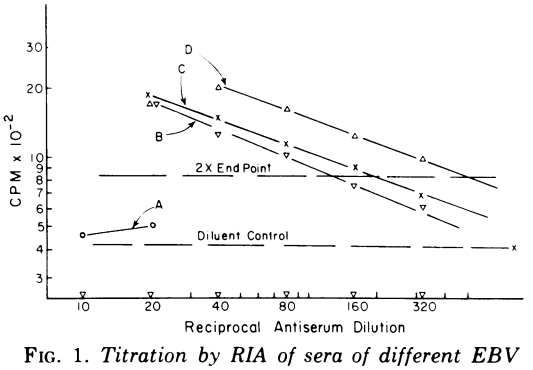

FIG. 1. *Titration by RIA of sera of different EBV antibody potencies. Each serum dilution was allowed to react with both HR1K and NC-37 cells. The reactivity of the sera was measured by reacting each dilution with ^{125}I-labeled guinea pig anti-human globulin. The radioactivity (counts per minute) recorded on the ordinate represented the net radioactivity adsorbed by the serum dilutions (HR1K minus NC-37). Serum end-point titers were the antiserum dilutions which adsorbed twice the radioactivity (counts per minute) adsorbed by the background controls (diluent). Symbols: serum A (○), titer = <10; serum B (▽), titer = 125; serum C (×), titer = 190; serum D (△), titer = 500.*

TABLE 1. *Human antibody titers to EBV as measured by RIA and IFA*

Serum no.	RIA[a, b]	IFA[a]
1219	500	200
1222	390	>400
1224 (S_1)	126	100
1224 (S_2)	190	100
1225 (S_1)	510	100
1225 (S_2)	210	100
1227	290	400
1231 (S_1)	420	100
1231 (S_2)	290	400
1232	<10	<10
1247	210	400
1248 (S_1)	<10	<10
1248 (S_2)	<10	<10
1249 (S_1)	22	200
1249 (S_2)	100	400
1250	205	100
1251 (S_1)	<10	<10
1251 (S_2)	<10	<10
1252	70	100

[a] Titers expressed as antiserum dilution factors.
[b] RIA titers obtained using HR1K as the positive cell line and NC-37 as the negative cell line.

the titers of six were at least twofold greater by RIA.

The reproducibility of the RIA procedure was previously reported by Hutchinson and Ziegler (9) and Ziegler et al. (16). The titers determined on successive days deviated less than twofold from the mean in seven trials. Although reproducibility was not extensively examined in this study, a single antiserum had an arithmetic mean titer of about 790 in three trials with a range of 520 to 1,000.

Because NC-37 and Raji cells contain small amounts of genomic material which are homologous with EBV-DNA, NC-37 or Raji control cells might contain some EBV-specific antigens. If this were true the use of NC-37 or Raji as the control cell line could obscure certain EBV-specific antibodies in the RIA procedure. Hence we tested five paired sera to determine their reactivity with EB-3 cells as the infected cell material and Raji and Molt-4 cells as the control cell material. The cell concentrations were adjusted so that the protein concentration of each was equal. The results (Table 2) showed that, with all the sera except one ($1S_2$), the adsorbed radioactivity was about the same with either Raji or Molt-4 cells as controls. The exceptional serum ($1S_2$) showed significantly higher adsorption of radioactivity by the Raji cells than by the Molt-4 cells. This phenomenon has not been investigated, but it may reflect differences in the antibody specificities of the serum. The results suggest that Molt-4 is the negative control cell material of choice.

TABLE 2. *Adsorption of EBV antibody-positive and negative sera to EB-3, Raji, and Molt-4 lymphoid cell lines*

Serum no.	Radioactivity adsorbed[a] (counts/min)		
	EB-3[b]	Raji[b]	Molt-4[b]
1			
S_1	1,724	1,403	1,465
S_2	4,734	4,282	3,020
2			
S_1	2.578	1,878	1,936
S_2	3,713	2,663	2,535
3			
S_1	1,358	1,944	2,122
S_2	4,356	2,270	1,926
4			
S_1	1,462	1,916	2,072
S_2	2,906	2,066	2,104
5			
S_1	1,594	1,392	1,177
S_2	4,340	2,204	2,186
Diluent	423	452	440

[a] Radioactivity (^{125}I-labeled anti-human globulin) adsorbed by lymphoid cells after reaction with a 1:10 dilution of human serum or diluent.
[b] Lymphoid cell lines: EB-3, EBV-infected cells; Raji, uninfected cells; Molt-4, uninfected cells.

The data also showed that with three of the convalescent sera ($1S_2$, $2S_2$, and $5S_2$), the ^{125}I-labeled anti-human globulin adsorbed by the control cells (Raji and Molt-4) increased significantly. The increased adsorption to the control cells may indicate antibody specificities for unknown antigens in the uninfected control cells. This adsorption of the sera to the uninfected cells has been treated as nonspecific reactivity in these experiments. However, the nature of the phenomenon is currently being investigated in our laboratory.

To show the specificity of the RIA, complete serum titrations were obtained for the five paired sera (preinfection or very early infection and postinfection) using EB-3 and Molt-4 cell lines. The results of RIA titrations showed complete agreement with those obtained by IFA (Table 3). By both procedures fourfold or greater increases in titers were observed for each of the serum pairs. Furthermore, the RIA and IFA titers of each of the S_2 serum specimens varied less than twofold. Thus, the results

TABLE 3. *RIA and IFA antibody titers to EBV of paired sera from patients with clinically diagnosed infectious mononucleosis*

Serum no.	RIA[a, b]	IFA[a]
1		
S_1	<10	10
S_2	150	200
2		
S_1	32	10
S_2	150	100
3		
S_1	<10	<10
S_2	300	400
4		
S_1	<10	<10
S_2	95	50
5		
S_1	<10	<10
S_2	570	500

[a] Titers expressed as antiserum dilution factors.
[b] RIA titers obtained using EB-3 as the EBV-infected cell line and Molt-4 as the uninfected cell line.

suggest that the sensitivity and specificity of the two assays are about the same.

The success of both RIA and IFA depends upon discrimination between specific and nonspecific binding of indicator antiglobulins (^{125}I-labeled antiglobulin in RIA and fluorescein-conjugated antiglobulin in IFA). In IFA optical filters reduce nonspecific fluorescence. However, trained technicians are required to perform both the technical procedures and the visual microscopy examination of each antigen-antibody reaction. The determination of titration end points entails acute interpretation of different colors and intensities of emitted fluorescence. Thus a technician of considerable experience and training is crucial to the success of the IFA procedure. In the proposed RIA method the tedious and time-consuming visualization of each reaction is not required. Each reaction is quantitated automatically using a gamma scintillation counter. The radioactivity adsorbed by the control cells is subtracted from the radioactivity adsorbed by the EBV-infected cells to obtain the net radioactivity specifically bound by antibodies reacting with the EBV antigens.

Scintillation counting equipment is relatively expensive; however, because of the increased routine use of RIA in diagnostic laboratories, the equipment is becoming more accessible for time sharing. The RIA described in this study is technically as simple as the IFA procedure, and it is at least as sensitive as IFA. Hence, if gamma scintillation counting equipment is available, the RIA procedure offers several distinct advantages. The use of the gamma scintillation counter for quantitation in the RIA method relieves the technician from tedious visual evaluation of specific fluorescence required in the IFA procedure. Thus, human judgment is eliminated and is replaced by quantitative and objective analytic determination of titration end points. In addition, the scintillation spectrometer presents RIA data in a form which is adaptable to electronic data processing.

We are now formulating a program for computer analysis of RIA data and calculation of serum end-point titers. This will effect partial automation of RIA. These advantages of the RIA procedure recommend it as a convenient, sensitive alternative to conventional serologic procedures.

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