

Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection

(infectious mononucleosis/chronic active infection)

WERNER HENLE*, GERTRUDE HENLE*, JAN ANDERSSON†, INGEMAR ERNBERG‡, GEORGE KLEIN‡, CHARLES A. HORWITZ§, GUNNAR MARKLUND¶, LARS RYMO||, CHRISTINA WELLINDER|| AND STEPHEN E. STRAUS**

*The Joseph Stokes, Jr., Research Institute at the Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, Philadelphia, PA 19104; †The University Clinic for Infectious Diseases, Danderyd Hospital, Danderyd, Sweden; ‡Institute for Tumor Biology, Karolinska Institute, Stockholm, Sweden; §Department of Laboratory Medicine, Mount Sinai Hospital, Minneapolis, MN 55404; ¶Department of Otorhinolaryngology, Sjödershuset, Stockholm, Sweden; ||Department of Medical Chemistry, Gothenburg University, Gothenburg, Sweden; and **National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT Five distinct Epstein-Barr virus (EBV)-determined nuclear antigens (EBNA-1 to EBNA-5) were recently identified. Antibody responses to these antigens could conceivably differ, and thus prove of serodiagnostic value, in EBV-associated disease processes. As a first step, murine or human cell lines transfected with appropriate EBV DNA fragments and stably expressing either EBNA-1 or EBNA-2 were used to determine the frequency and time of emergence of antibodies to these two antigens in the course of acute and chronic infectious mononucleosis (IM) and to assess their titers in so-called chronic active EBV infections. Following IM, antibodies to EBNA-2 arose first and, after reaching peak titers, declined again in time to lower persistent or even nondetectable levels. Antibodies to EBNA-1 emerged several weeks or months after anti-EBNA-2 and gradually attained the titers at which they persisted indefinitely. The ratios between the anti-EBNA-1 and anti-EBNA-2 titers therefore were generally well below 1.0 during the first 6-12 months after IM and turned to well above 1.0 during the second year. In clear cases of chronic IM, the inversion of this ratio was delayed or prevented. In the less well-defined chronic EBV infections, low ratios were observed in only some of the patients. Because many of these illnesses were not ushered in by a proven IM and often showed EBV-specific antibody profiles within the normally expected range, a causal role of the virus in these cases remains doubtful.

Epstein-Barr virus (EBV)-encoded antigens are divided into those associated with the viral replicative cycle [i.e., the viral capsid antigen (VCA) and the early antigens (EA) of the diffuse (D) and restricted (R) varieties] and those expressed in latently infected B lymphocytes, the EBV-determined nuclear antigen (EBNA) (1). Both categories contain, in addition, distinct EBV-induced cell membrane antigens. Antibodies to VCA and the EA components emerge during the late incubation period or in the course of the acute phase of infectious mononucleosis (IM), whereas antibodies to EBNA arise only weeks or months after onset of the disease, indicating that the two sets of antigens become available for stimulation of antibody responses from separate sets of cells under different circumstances (2). Similarly divergent antibody responses to VCA and EA components on the one hand, and to EBNA on the other, may also be seen following activation of persistent latent EBV infections by immunosuppressive disease or therapy (3).

The determination of antibodies to VCA, D, R, and EBNA, as well as identification of the immunoglobulin class of the antibodies have been useful in the serodiagnosis of various EBV-induced diseases (reviewed in ref. 1). The antibody profile evoked by IM is distinct from the profiles seen in EBV-associated Burkitt lymphoma or nasopharyngeal carcinoma, respectively, and all three profiles again differ from the antibody pattern observed in healthy individuals after a long-past primary EBV infection. Unfortunately, the antibody profiles typical of Burkitt lymphoma or nasopharyngeal carcinoma may emerge only with advancing tumor burden, and patients with certain other malignant diseases or nonmalignant lymphoproliferative or immunosuppressive conditions may occasionally develop antibody profiles comparable to those of Burkitt lymphoma or nasopharyngeal carcinoma (3). The discovery and utilization of additional EBV-encoded antigens might further improve the specific serodiagnosis of EBV-related diseases.

EBNA is now known to be a complex of several antigens. First EBNA-1 and EBNA-2 were separated (4-8), followed by EBNA-3 (9-11), and most recently EBNA-4 (11) and EBNA-5 (12). EBNA-1 appears to be required for maintenance of the virus in episomal form (13). The function of EBNA-2 is still unknown, but the fact that EBNA-2 deleted viral variants obtained from P3JHR-1 or Daudi cultures do not activate B lymphocytes suggests that this antigen is involved in the primary B-cell activation (14, 15). The genes for EBNA-1 and EBNA-2 have been identified in the EBV genome (5, 16-19). We have transfected DNA fragments containing the coding exons for EBNA-1 or EBNA-2 into mouse fibroblasts or human lymphoid cell lines to produce cells that stably express these antigens (ref. 16; C.W., A. Ricksten, and L.R., unpublished data). Practically all cells in the transfected cultures express either EBNA-1 or EBNA-2. Such EBNA-1- or EBNA-2-producing cell lines were used for the detection and titration of antibodies to these two components and to determine the frequency and time of their emergence in the course of primary EBV infections and their levels in so-called chronic active EBV infections.

MATERIALS AND METHODS

Sera. All sera had been collected for earlier studies concerning IM and chronic IM-like illnesses and had been stored at -20°C until used. Sera from IM patients had been

examined previously for the transient emergence of IgA antibodies to VCA in the early acute phase or of IgG antibodies to R in late convalescence (20–25). Some of the IM patients had participated in open or double-blind placebo-controlled studies on the effectiveness of tinidazole or acyclovir therapy, respectively. The sera of patients with chronic IM-like illnesses were derived mainly from an ongoing study carried out at the National Institutes of Health, some of whom were previously described (26). The chronic illnesses were characterized by various degrees of fatigue, malaise, lymphadenopathy, sore throat, and other constitutional symptoms. For the present analysis, these patients were divided into three groups. Group 1 comprises patients who in the wake of a confirmed IM developed numerous successive, often life-threatening, complications over the course of many years accompanied by excessively high IgG antibody titers to VCA and to D ($\geq 1:10,240$) and often also high titers of IgA antibodies to these antigens, whereas antibodies to EBNA remained at normal or subnormal levels (27, 28). There is little doubt that EBV caused these rare cases. Group 2 includes patients whose chronic illnesses were ushered in by a proven IM in only some but by no means all cases. Their IgG antibody titers to VCA were elevated (1:640 to 1:5120) and their anti-EA titers, directed mostly against R, rarely against D, ranged from 1:80 to 1:640, but the anti-EBNA levels were moderate or nondetectable. Group 3, by far the largest, is clinically indistinguishable from group 2. The EBV-specific antibody profiles of these patients remained well within the range normally expected years after a primary EBV infection, except that a few lacked antibodies to EBNA. Data supporting an association of EBV with the illnesses of the patients in groups 2 and 3 are not compelling, but elevated antibody titers render such an association for group 2 more likely than for group 3. That other causes for these chronic complaints exist became clearly evident from a small fourth group of patients who had similar complaints as the patients in groups 2 and 3 but were as yet EBV seronegative (unpublished observations).

EBV-Specific Serology. Antibodies to VCA, D, and R were determined by indirect immunofluorescence and antibodies to EBNA by anti-complement immunofluorescence as described (29, 30). Murine 3T3 fibroblasts transfected with the *Bam*HI K fragment of B95-8 EBV DNA and designated 3T3/pBK cells (8) served as a source of EBNA-1. Human DG75 cells transfected with a DNA fragment containing the *Bam*HI WYH region of EBV DNA and referred to as

DG75/pE Δ A6 cells (C.W., A. Ricksten, and L.R., unpublished results) served as a source of EBNA-2. Raji cells containing all known EBNA components but EBNA-5 (12) and BJAB cells containing none of them served as positive and negative controls, respectively. Raji, BJAB, and DG75/pE Δ A6 cells do not attach to the culture vessels, but the 3T3/pBK cells grow attached to glass or plastic. They were dislodged by trypsin (0.25%) and washed before cell smears were made in the same manner as described elsewhere in detail for lymphoblastoid cells (31). Care was taken to obtain near monolayers and to prevent piling up of the cells by spreading a small drop containing $\approx 3 \times 10^5$ cells over 6×30 mm coverslips. The smears were allowed to dry rapidly in the air current of a laminar flow hood and, when seemingly dry, were kept for 2 hr at room temperature before being fixed for 5 min in a precooled (-20°C) equal mixture of acetone and methanol. After fixation, the smears were kept in plastic boxes at -20°C until used.

RESULTS

Anti-EBNA-1 and Anti-EBNA-2 Responses in IM. Fig. 1 and Table 1 present the EBV-specific serologic results obtained with serial sera from numerous IM patients. The diagnosis was confirmed in each case by demonstration of IgM antibodies to VCA and initial absence of antibodies to EBNA, by the detection of IM-specific heterophil antibodies in nearly all, and by the presence of antibodies to the D component of the EA complex in $\approx 80\%$ of the patients. The sera are grouped according to the time they were collected after onset of disease or admission to inpatient or outpatient services. Each dot in the figure presents the results obtained with a given serum, the anti-EBNA-1 titer being found on the abscissa and the anti-EBNA-2 titer on the ordinate. Practically all EBNA-specific antibodies that were detected by the use of Raji cell smears within the first 3 months were directed against EBNA-2. Nearly 75% of the sera collected during this period still revealed VCA-specific IgM at titers ranging from 1:10 to 1:80 (Table 1); anti-D was detected in $\approx 60\%$ as the sole antibody to the EA complex or it was exceeded in titer or replaced by anti-R so that almost one-half of the patients showed antibodies to R. The next group of sera, collected mainly 6 and 12 months after onset of IM, revealed the emergence in the majority of the patients of low levels of antibodies to EBNA-1 that in most instances were exceeded

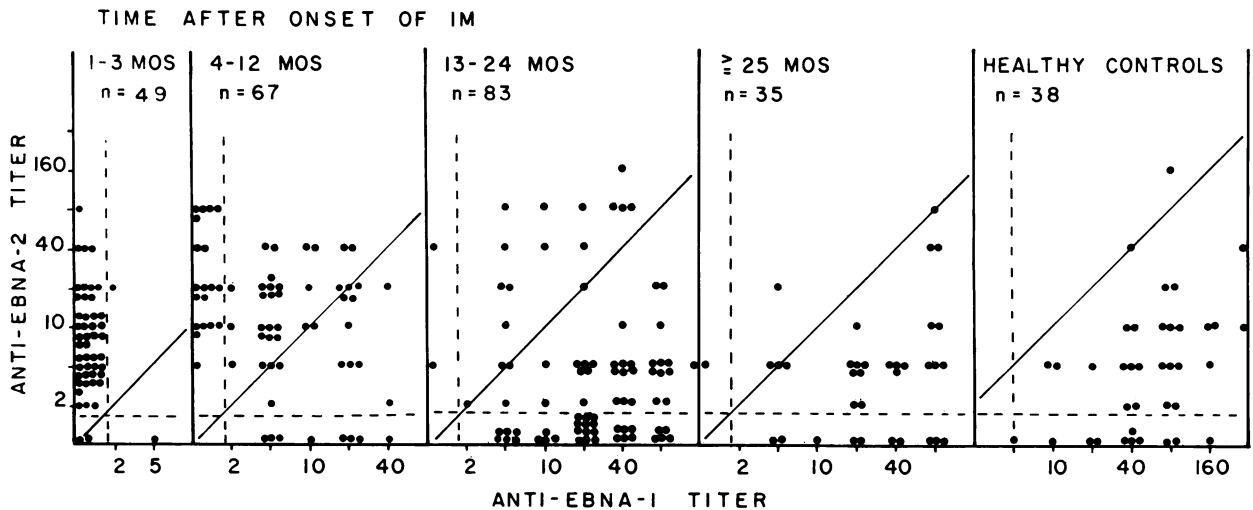


FIG. 1. Antibody titers to EBNA-1 and EBNA-2 at increasing intervals after onset of infectious mononucleosis. Each dot shows the results on one serum, the anti-EBNA-1 titer is found on the abscissa, and the anti-EBNA-2 titer on the ordinate. If the anti-EBNA-2 titer exceeds the anti-EBNA-1 titer, the dot falls above the diagonal line.

Table 1. Serological parameters at various times after onset of IM

	Months after onset					Healthy controls (n = 38)
	0 (n = 74)	2-3 (n = 44)	4-12 (n = 65)	13-24 (n = 83)	25-48 (n = 35)	
IgM anti-VCA						
% positive	100.0	73.1	0.0	0.0	0.0	0.0
Range of titers	80-640	10-80	<10	<10	<10	<10
Anti-EA						
% positive	81.8	88.5	87.8	60.9	39.4	30.0
Range of titers	10-320	10-160	10-160	10-80	10-80	10-40
Anti-D						
% positive	81.8	57.7	10.2	13.0	0.0	0.0
Range of titers	10-320	10-160	10-160	10-40	<10	<10
Anti-R						
% positive	—*	46.2	83.7	47.9	39.4	30.0
Range of titers	—	10-160	10-160	10-80	10-80	10-40
Anti-EBNA (Raji)						
% positive	0.0	97.7	100.0	100.0	100.0	100.0
Anti-EBNA-1						
% positive	0.0	4.5	73.8	97.6	97.1	100.0
Geometric mean titer	<2	<2	5.3	21.2	24.8	48.2
Anti-EBNA-2						
% positive	0.0	93.2	87.9	60.2	71.4	71.1
Geometric mean titer	<2	7.3	11.6	4.1	4.3	3.9
Ratio anti-EBNA-1/anti-EBNA-2						
≤1.0	—	97.7	76.9	22.9	17.1	5.3
>1.0	—	2.3	23.1	77.1	82.9	94.7

*Anti-R can be measured only when exceeding anti-D in titer.

by the anti-EBNA-2 titers. In a few cases, however, antibodies to EBNA-2 had become nondetectable (<1:2). During this period, IgM antibodies to VCA were no longer measurable and anti-D was uncommon, whereas anti-R was now detected in >80% of the cases. During or after the second year after IM, practically all sera had antibodies to EBNA-1, which in time gradually exceeded anti-EBNA-2 in titer in an increasing proportion of cases. Indeed, antibodies to EBNA-2 were no longer detected in about one-third of the individuals 13 or more months after IM. With progression of time, the incidence and titers of antibodies to EA, directed almost exclusively to R, showed a gradual decline. The ratio between most paired individual or the geometric mean titers of anti-EBNA-1 and anti-EBNA-2 was well below 1.0 during the first year after onset of IM and turned to well above 1.0 during or after the second year. This reversal in the ratio was noted as early as 6 months and as late as 2 years after onset of IM.

Chronic IM or IM-Like Illnesses. Fig. 2 presents the anti-EBNA-1 and anti-EBNA-2 titers of the three seropositive groups of patients described in *Materials and Methods*. The chronic IM patients in group 1 with excessively high anti-VCA and anti-D titers (Fig. 2 *Lower*) had, with a single exception, anti-EBNA-2 titers that equalled or exceeded the anti-EBNA-1 levels (Fig. 2 *Upper*). Three of these patients, in fact, had no detectable anti-EBNA-1, as noted earlier (28, 32). The patients with less well-defined illnesses in groups 2 and 3 had, with rare exceptions, antibodies to both EBNA-1 and EBNA-2, but the anti-EBNA-2 titers were found to be equal to or higher than the anti-EBNA-1 titers in 23% and 30% of the cases, respectively. Only 2 of the 69 patients lacked antibodies to EBNA-1 and four lacked antibodies to EBNA-2. All healthy seropositive controls in this particular series had antibodies to EBNA-1, but nearly 30% lacked detectable antibodies to EBNA-2 and in none did the titer of anti-EBNA-2 equal or exceed the titer of anti-EBNA-1. These results suggest that, in some cases of chronic active IM or IM-like illnesses, the change from dominant anti-EBNA-2 to dominant anti-EBNA-1 may be delayed or prevented.

Efforts to correlate the individual titers or ratios between antibody titers to EBNA-1 and EBNA-2 to the past history and persisting signs and symptoms of the patients in groups 2 or 3 were unsuccessful. There were no clear associations between duration or nature of illnesses, anti-EA titers, or history of IM with the results of the anti-EBNA-1 or anti-EBNA-2 tests.

DISCUSSION

The results have shown that the sum total of anti-EBNA titers obtained with Raji cell smears is always closely matched by either the anti-EBNA-1 or the anti-EBNA-2 titer depending on which of the two is dominant. Antibodies to the other EBNA components thus may normally fail to affect the overall anti-EBNA titer.

While this manuscript was in preparation, a report was published showing that antibodies to EBNA-1 appeared long after anti-EBNA had become detectable by the use of Raji cell smears (33). As shown here, the antibody production to EBNA-1 and to EBNA-2 in the course of IM appears to be subject to an orderly progression. Anti-EBNA-2 emerges first, reaches peak titers, and then declines to a lower persistent level or even becomes nondetectable in about one-third of the cases. Anti-EBNA-1 arises long after anti-EBNA-2, increases gradually in titer to exceed in time the level of anti-EBNA-2, and then persists indefinitely at the ultimately attained plateau. Thus, within the first year after IM, the ratio between the anti-EBNA-1 and anti-EBNA-2 titers is usually well below 1.0, whereas later the ratio turns generally to well above 1.0. The switch from dominant anti-EBNA-2 to dominant anti-EBNA-1 titers occurs in individual cases over a long span of time. Could a delay in the inversion of the ratio or a failure of its occurrence be associated with persistent intermittent or continuous clinical complaints?

Affirmative evidence was provided by the patients presented as group 1, who in the wake of a proven IM developed prolonged, often life-threatening, complications as well as

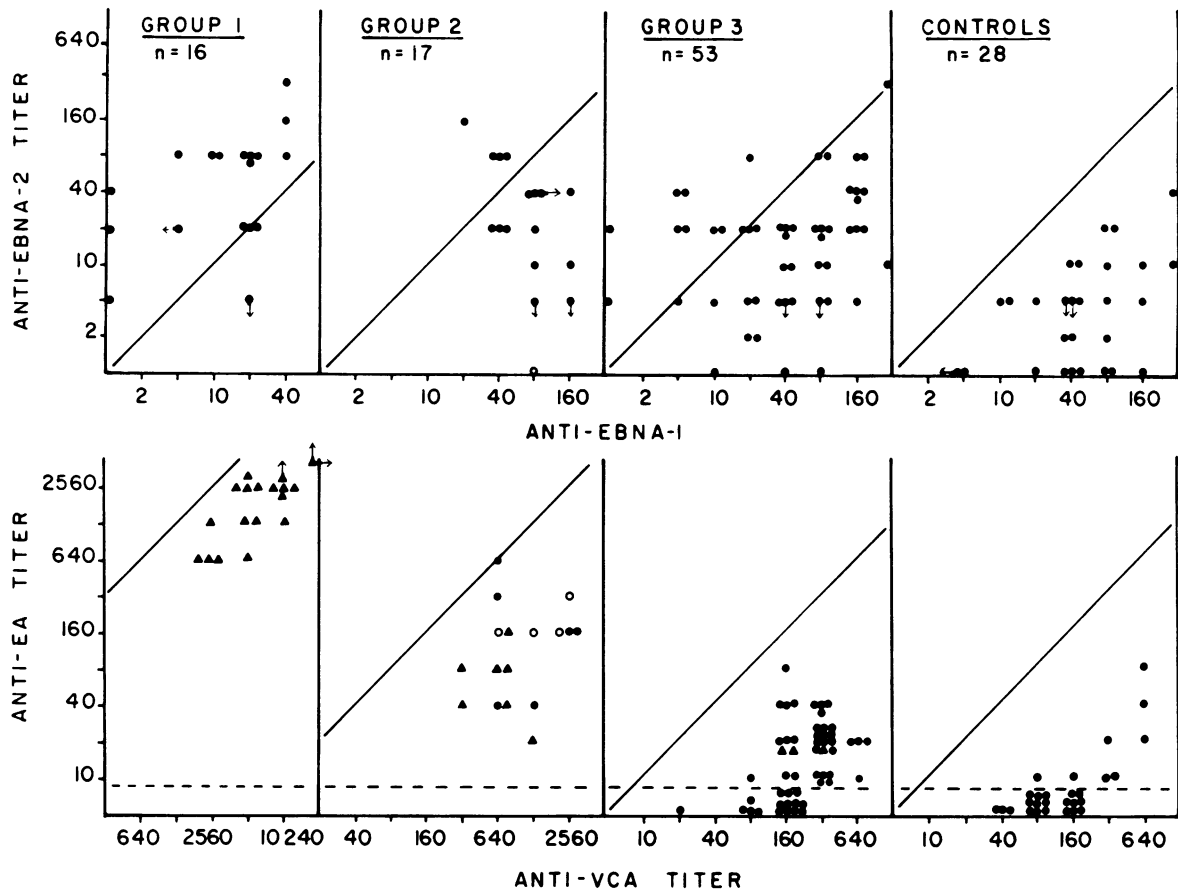


FIG. 2. Antibody titers to EBNA-1 and EBNA-2 (*Upper*) in patients with chronic infectious mononucleosis (group 1) or chronic IM-like illnesses (groups 2 and 3). (*Lower*) The anti-VCA titers are plotted against the anti-EA titers. ▲, Anti-D dominant; ○, anti-D exceeded by anti-R; ●, anti-R only.

excessively high antibody titers to VCA and to D (27, 28). All but one of the patients in this category either lacked antibodies to EBNA-1, as noted earlier (32), or showed dominant anti-EBNA-2 titers. Thus, demonstration of anti-EBNA-1/anti-EBNA-2 ratios of <1.0 more than 2 years after onset of IM indicates a defective control of the persistent latent EBV carrier state that regularly ensues after primary EBV infections. Also, some of the patients in groups 2 and 3 who suffered from less well-defined so-called chronic active EBV infections (26, 34, 35) showed an anti-EBNA-1/anti-EBNA-2 ratio of <1.0 . Because some of these illnesses were not ushered in by a bonafide IM, a ratio of <1.0 might not have been due to an arrest in the progression of the early immune responses but rather the result of an activation of the latent persistent viral carrier state by some immunosuppressive event—e.g., another infectious disease. The subset of patients with Hodgkin disease or non-Hodgkin lymphomas who develop unusually high antibody titers to VCA and to D or R (36) or patients with the acquired immunodeficiency syndrome or genetic immune defects show indeed a high incidence of anti-EBNA-1/anti-EBNA-2 ratios below 1.0 (unpublished data). Attempts to correlate low ratios among the patients of groups 2 and 3 with specific clinical observations failed to meet with success.

The question why antibodies to EBNA-2 emerge well ahead of anti-EBNA-1 cannot be answered at present. Obvious explanations would be that EBNA-2 is produced earlier, or in larger amounts, or is a more potent antigen than EBNA-1. Both antigens appear, however, almost simultaneously in EBV-transformed B lymphocytes and are synthesized in seemingly comparable quantities, judging by the degrees of immunofluorescence they yield or by immune blot

analysis (11, 13, 37). It is unknown, though, whether and in what amounts EBNA components are synthesized in oropharyngeal epithelial cells, which are thought to be the initial targets of the virus in primary EBV infections (38). The assumption that EBNA-2 is a more potent antigen than EBNA-1 also offers an unresolved problem. Why should production of antibodies to EBNA-2 decline or even cease in time, whereas antibodies to the “weaker” EBNA-1 ultimately become dominant and persist? Could this be a matter of a difference in presentation of the antigens early in the course of a primary EBV infection, when a large number of B lymphocytes are growth transformed, as compared to later when only a few such cells circulate? These problems may be resolved only after successful cultivation of the appropriate fully permissive target cells involved in the primary EBV infection to permit detailed study of the temporal and quantitative aspects of the emergence of various antigens after exposure to the virus.

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