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Use of Enzyme-Linked Immunosorbent Assays with Chimeric Fusion Proteins To Titrate Antibodies against Epstein-Barr Virus Nuclear Antigen 1

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Two new enzyme-linked immunosorbent assays (ELISAs) with chimeric fusion polypeptides for the detection of human antibodies specific to Epstein-Barr virus nuclear antigen 1 (EBNA-1) are described. One is an indirect ELISA with affinity-purified β-galactosidase–EBNA-1 fusion protein as the antigen. The other is a "sandwich" assay based on the use of anti-β-galactosidase antibody to capture β-galactosidase–EBNA-1 fusion proteins in bacterial extracts. A good correlation was shown between antibody titers determined by the ELISA with the EBNA-1 fusion proteins and those determined by a conventional anticomplement immunofluorescence test which is being widely performed with Raji cells for the purpose of research and clinical diagnosis. The advantage of the ELISAs for seroepidemiologic studies on Epstein-Barr virus was demonstrated by sensitive detection of marginal immunoglobulin G antibody to the EBNA-1 domain in serum samples from patients with infectious mononucleosis.

Epstein-Barr virus (EBV) causes infectious mononucleosis (IM) and is considered associated with nasopharyngeal carcinoma and African Burkitt's lymphoma (2, 6, 11, 12). EBV infects human B lymphocytes and establishes a so-called latent infection in them, inducing their infinite proliferation. EBV nuclear antigen (EBNA) is found in the proliferating cells by the anticomplement immunofluorescence (ACIF) test (23). The immune responses to EBV-specific antigens in acute IM are characterized by raised antibody titers to viral capsid antigen (VCA) and undetectable titers of antibodies against EBNA (10). A decrease in antibody response to EBNA was suggested to be associated with defective T-cell function (1, 20, 28). EBNA, which was originally defined serologically (10, 23), has recently been verified to be composed of six distinctive polypeptides, and the components have been designated EBNA-1, EBNA-2, EBNA-3, EBNA-4 (or EBNA-3B), EBNA-5 (or EBNA-LP), and EBNA-6 (or EBNA-3C) (3, 16, 25). These findings necessitate molecular serological studies on "EBNA" to examine whether antibody responses to individual EBNA polypeptides have diagnostic and/or prognostic significance.

Recently, antibody responses to EBNA-1 and EBNA-2 have been analyzed separately by ACIF tests with EBNA-1-expressing and EBNA-2-expressing cell lines which were established by transfection of recombinant DNAs constructed for their expression (13, 27). However, ACIF tests are relatively laborious, and determination of anti-EBNA antibody titers by these tests is occasionally hindered by interference from antinuclear autoantibodies in human sera, especially from patients with autoimmune diseases.

We have recently reported the production and characterization of chimeric fusion proteins which were constituted from β-galactosidase and domains of EBNA-1, EBNA-2, EBNA-3, EBNA-4, or EBNA-6 (15). We also prepared polyclonal antibodies against these fusion proteins and monoclonal antibodies to EBNA-1 (14, 15). In this study, we developed two kinds of enzyme-linked immunosorbent assays (ELISAs), an indirect ELISA and a "sandwich" ELISA, for detection of antibodies to EBNA-1 polypeptide by using the fusion proteins as specific antigens.

MATERIALS AND METHODS

β-galactosidase-EBNA fusion proteins. The DNA construction, production, and properties of β-galactosidase–EBNA-1 fusion proteins used in this report were described previously (15). The K2 and SII fusion proteins contain amino acids 451 to 641 and 451 to 618 of EBNA-1, respectively. Bacterial extracts were prepared as described previously (15). Briefly, *Escherichia coli* lysogenized with the recombinant λgt11 bacteriophage was grown at 32°C to 10^8 cells per ml. The cells were incubated at 45°C for 20 min, cultured at 39°C for 1.5 h after the addition of IPTG (isopropyl-1-thio-β-galactopyranoside) to 0.1 mM, and harvested by centrifugation. The cells were treated with lysozyme, the nonionic detergent CHAPSO (3[(3-cholamidiopropyl)-dimethylammonio]-2-hydroxypropane sulfonate; Dojin, Kumamoto, Japan), freeze-thawed, and sonicated. The crude extracts, which were prepared by centrifugation at 60,000 × *g* for 30 min, were partially purified by precipitation with ammonium sulfate (30% saturation). The precipitates were resuspended in phosphate-buffered saline (PBS) and used as antigens for the sandwich ELISA. The fusion proteins constituted from 20 to 40% of the bacterial extracts.

Immunoaffinity purification of SII(EBNA-1) fusion protein. The SII fusion protein in the bacterial extract was purified by DEAE-cellulose chromatography. A fraction bound to the column with low-salt buffer (20 mM Tris-HCl [pH 7.5], 0.2 M NaCl) was eluted with high-salt buffer (20 mM Tris-HCl [pH

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7.5], 0.3 M NaCl), dialyzed against PBS containing 0.01% Tween 20, and applied to a Sepharose 4B column which was coupled with anti-EBNA-1 monoclonal antibody E1-297 (14). The protein that bound to the immunoaffinity column was eluted with 0.1 M glycine-HCl (pH 2.5), dialyzed against PBS, and used as the antigen for the indirect ELISA.

Indirect ELISA with the SII fusion protein. Each well of microtiter plates (Immunoplate II; Nunc, Roskilde, Denmark) was coated with 100 μl of PBS containing 0.1 μg of the affinity-purified SII fusion protein at 4°C for 24 h and blocked with 300 μl of PBS containing 2% skim milk. Human serum samples were diluted 1:200 with the 2% skim milk solution containing 1 μg of the extract from bacteria lysogenized with λgt11, which codes for β-galactosidase, per ml and incubated at 37°C for 1 h and at 4°C for 12 h. The diluted human serum solutions were added to the wells (100 μl per well) and incubated at 37°C for 1 h. After the reaction and a wash with PBST (PBS containing 0.05% Tween 20), horseradish peroxidase (HRPO)-conjugated goat anti-human immunoglobulin G (IgG; heavy and light chain) antibody (MBL, Nagoya, Japan) was added and incubated at 37°C for 1 h, and then 100 μl of development solution (0.04% ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfate)], 2.0% citrate buffer [pH 4.0], 0.05% H_2O_2) was added and incubated at room temperature for 20 min. The reaction was stopped by addition of 100 μl of 0.02% sodium azide, and the OD_{415} of the reaction solution was measured.

Anti-β-galactosidase IgG and its F(ab')$_2$ fraction. New Zealand White rabbits were immunized with β-galactosidase which was purified from *E. coli* (Sigma). Antibodies were fractionated with ammonium sulfate, and the IgG fractions were isolated by DEAE-cellulose column chromatography. The F(ab')$_2$ of the isolated IgG antibodies was prepared by digestion with immobilized pepsin (17).

Sandwich ELISA with fusion proteins. The sandwich ELISA (5) was modified and examined by taking advantage of the two, common (β-galactosidase) and specific (EBNAs), antigenic domains which constitute the individual fusion proteins. The assay was essentially composed of the following three steps: (i) capture of the fusion protein in bacterial extract by anti-β-galactosidase antibody, (ii) binding of antibodies in human serum samples to the captured fusion protein, and (iii) detection of the bound human antibodies. Microtiter plates (Immunoplate II) were coated with a solution containing rabbit anti-β-galactosidase IgG F(ab')$_2$ fraction in PBS (100 μl per well) at 4°C for 24 h. The plates were blocked by incubation with 300 μl of PBS containing 2% skim milk at 4°C overnight. Bacterial extract containing fusion protein or β-galactosidase was added as the specific or negative-control antigen, respectively, and incubated at 37°C for 1 h. After the plate was washed with PBST, 100 μl of human serum diluted with the 2% skim milk solution was added and incubated at 37°C for 1 h. After a wash with PBST, 100 μl of HRPO-conjugated goat anti-human IgG (Fc-specific) antibody (240 U of peroxidase activity per ml; peroxidase/antibody ratio, 0.9; ICN Immuno Biologicals) was added and incubated at 37°C for 1 h. After another wash with PBST, the plates were incubated with 100 μl of development solution per well at room temperature for 40 min, and the reaction was stopped by addition of 0.02% sodium azide. OD_{415} values specific to EBNA domains were obtained by subtracting the OD_{415} values for the β-galactosidase extract from those for the fusion proteins, and the result is referred to as the ΔOD_{415}.

Human sera. The diagnosis of IM was done at one hospital by clinical symptoms and laboratory examinations according to Evan's criteria (7). General clinical signs are summarized as fever, sore throat (exudative pharyngotonsillitis), lymphadenopathy (symmetric cervical lymphadenopathy), and hepatosplenomegaly. Laboratory examinations showed atypical lymphocytosis (68% of IM patients), peripheral leukeocytosis (50% of IM patients), and liver dysfunction (high levels of serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, and lactic dehydrogenase [100% of IM patients]). Two of the 19 serum samples from IM patients tested in this study were collected at 1.5 and 2 months after onset, respectively, and the rest (17 serum samples) were collected at 3 days to 3 weeks poston-set (mean ± standard deviation [SD], 12.9 ± 6.4 days). Serologic examination of antibody responses to EBV antigens was done by conventional methods (10, 23).

RESULTS

Development of indirect ELISA with affinity-purified EBNA-1 fusion protein as the antigen. Since SII was one of the most abundantly produced EBNA-1 fusion proteins in *E. coli* (15), a large amount of the SII fusion protein was purified by DEAE-cellulose chromatography and monoclonal antibody-coupled Sepharose chromatography. Approximately 1 mg of the affinity-purified protein was obtained from 1 liter of bacterial culture (data not shown).

The indirect ELISA was attempted by binding the purified SII to the solid phase and monitoring the detection of human antibodies bound to the immobilized protein. Sera were preabsorbed with the bacterial extract containing β-galactosidase. The efficiency of the preabsorption was examined by comparing the OD_{415} after the reaction of serum samples which had high antibody titers against β-galactosidase with and without preabsorption. The OD_{415} values of the serum samples at a 1:200 serum dilution after incubation in the presence of 0.1 and 1 μg of the extract per ml were 0.1 to 0.2 and less than 0.1, respectively, although those in the absence of extract were 0.2 to 0.4 (data not shown).

The OD_{415} values of serum samples from the following Japanese individuals were measured by the ELISA with the SII fusion protein at the 1:200 serum dilution: 1 anti-VCA antibody-negative healthy adult and 15 anti-VCA antibody-negative infants from 1 to 3 years old (group A); 16 anti-VCA antibody-positive healthy infants from 1 to 3 years old (group B); 38 anti-VCA antibody-positive healthy adults (group C); and 17 IM patients (group D) (Fig. 1). Since less than 2% of Japanese adults are EBV seronegative, we had to obtain and test EBV-negative sera from infants. Approximately half of the infants from 1 to 3 years old in Japan were seronegative for EBV (data not shown). The average ± SD OD_{415} value for anti-VCA antibody-negative serum samples (group A) was 0.086 ± 0.059, while for the anti-VCA antibody-positive serum samples (groups B and C), the average OD_{415} ± SD was 1.153 ± 0.474. The average ± SD for serum samples from IM patients (group D) was 0.190 ± 0.126. The reaction and OD_{415} measurement of serum samples from three seropositive and one seronegative individual were tested three times to examine the reproducibility. The means ± SD were 1.58 ± 0.09, 0.74 ± 0.05, 0.41 ± 0.05, and 0.01 ± 0.02, respectively, indicating that the ELISA worked reproducibly. It should be noted that OD_{415} values specific to the carrier domain of the fusion protein, i.e., β-galactosidase, were measured with affinity-purified β-galactosidase in parallel in these analyses. The average ± SD for all 87 serum samples was 0.077 ± 0.086, indicating that the preabsorption of anti-β-galactosidase antibodies with the bacterial extract

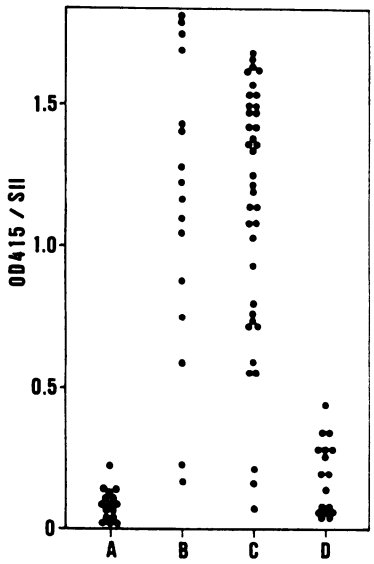

FIG. 1. Indirect ELISA with the affinity-purified SII(EBNA-1) fusion protein as the antigen. Anti-SII antibody titers in the following groups of serum samples, which were preabsorbed with β-galactosidase, were measured in terms of OD_{415} values at a serum dilution of 1:200. Group A, 16 anti-VCA antibody-negative healthy individuals; group B, 16 anti-VCA antibody-positive healthy infants from 1 to 3 years old; group C, 38 anti-VCA antibody-positive healthy adults; group D, 17 IM patients. The *y* axis indicates OD_{415} values.

containing β-galactosidase was done properly. Thus, the OD_{415} values measured by the indirect ELISA with SII described above were considered EBNA-1 domain specific.

Optimized conditions for the sandwich ELISA with fusion proteins. In addition to the indirect ELISA, we developed a sandwich ELISA in which anti-β-galactosidase antibody was used to capture the β-galactosidase-EBNA fusion proteins in bacterial extracts.

The $F(ab')_2$ fraction of rabbit anti-β-galactosidase IgG was tested as the coating antibody in place of the intact IgG molecule because there was some immune reactivity between the coated rabbit anti-β-galactosidase IgG and the HRPO-conjugated goat anti-human IgG (Fc-specific) antibodies (data not shown). There was no significant immune reactivity of the $F(ab')_2$ fraction of rabbit IgG with the HRPO-conjugated goat antibody (Fig. 2A, broken line with asterisks). Then, we optimized the conditions for this assay as follows. First, a range of concentrations of the anti-β-galactosidase IgG $F(ab')_2$ fraction for coating were examined (Fig. 2A). The K2 fusion protein and HRPO-conjugated anti-human IgG (Fc-specific) antibody were saturated in the test. Human serum TWM1, with a high antibody titer to EBNA-1, was used at a dilution of 1:5,000 (open circles) and 1:25,000 (solid circles). There was a linear correlation between the concentration of the coating anti-β-galactosidase IgG $F(ab')_2$ fraction and the measured OD_{415} value in the range above 6 ng of the $F(ab')_2$ fraction per well. Second, the reaction's dose dependence on the fusion protein was examined. The OD_{415} reached saturation with 2 μg of the extract protein, approximately 20% of which was K2 fusion protein (Fig. 2B). Third, a range of concentrations of the HRPO-conjugated anti-human IgG (Fc-specific) antibody were tested, and a 1,000-fold dilution was used (Fig. 2C).

Based on the experiments described above, the optimum assay conditions were set as the following: 12 ng of rabbit anti-β-galactosidase IgG $F(ab')_2$ per well, 2 μg of the K2 fusion protein extract per well, and a 1,000-fold dilution of HRPO-conjugated anti-human IgG antibody. Under these conditions, titration of specimens of human serum were carried out (Fig. 2D to I). Figure 2D depicts the titration curve with anti-VCA antibody-negative serum OKA582. Anti-VCA antibody-positive sera TWM122 (Fig. 2E), TWM125 (Fig. 2F), and TWM21 (Fig. 2G), all from healthy individuals, had anti-EBNA titers of less than 10, 40, and 80, respectively, as determined by the ACIF test with Raji cells. There was a good linearity between OD values and dilutions of sera in the OD range from 0.2 to 1.5. The extract from bacteria lysogenized with λgt11 which coded β-galactosidase was used as the negative control antigen (solid circles in Fig. 2D to I).

Significant antibody titers to β-galactosidase itself were detected in only 6 of 110 samples tested. After the reaction of 200-fold-diluted serum samples with the control β-galactosidase, the OD of one of the six sera was 1.9 and that of the other five ranged from 0.40 to 0.57. The mean ± SD of OD values measured in the same fashion with the rest of the test sera was 0.15 ± 0.10. Hence, the ELISA titer was defined as the highest dilution of serum that gave a ΔOD_{415} (difference in OD_{415} between the EBNA fusion protein and β-galactosidase) of 0.3 or higher. Next, serum samples from patients with autoimmune diseases, which had antinuclear antibodies hindering the ACIF test (data not shown), were examined. TWM75 (Fig. 2H) and TWM96 (Fig. 2I) were from a patient with Sjögren's syndrome and from a Sjögren's syndrome patient who also had systemic lupus erythematosus, respectively. The reactions of these serum samples from patients with autoimmune diseases in the ELISA were specific to the EBNA-1 domain.

The same serum samples that were used in the indirect ELISA and two more samples from patients with IM were analyzed by the sandwich ELISA as described above (Fig. 3A). The average ± SD ΔOD_{415} values for 16 anti-VCA antibody-negative serum samples (group A) was 0.080 ± 0.085. For 54 anti-VCA antibody-positive serum samples (group B and C), the average ± SD was 1.27 ± 0.57. Only three anti-VCA antibody-positive serum samples had undetectable titers of anti-EBNA-1 antibodies at the dilution tested. One of the three serum samples, TWM38 (group C), showed an undetectable (lower than 10) anti-"EBNA" titer in the ACIF test with Raji cells but had anti-EBNA-2 antibodies which were detected both by the ELISA with the H7(EBNA-2) fusion protein and by immunoblotting analysis with a lysate of Raji cells or the EBNA-2 fusion protein as the antigen (data not shown). The average ± SD for 19 serum samples from patients with IM (group D) was 0.046 ± 0.136. The ΔOD_{415} values at this serum dilution (1:200) were compared with their ELISA titers (Fig. 3B). There was a good correlation (relative coefficient, 0.96) in the range below 1.5 OD_{415} in the two measurements, but the ΔOD_{415} values of serum samples which had ELISA titers above 3,200 were saturated.

We tested reproducibility by repeating the assay twice and showed that 12 of the 38 serum samples (group C) showed a twofold-dilution difference in titer and the rest (26 serum samples) had exactly the same titers. Two serum samples, TWM1 and YNI, were tested five times to examine the reproducibility further, and their titers were 12,800 and 3,200 respectively, every time.

Comparison of ELISAs and conventional ACIF test. A comparison of measurements by the indirect ELISA and

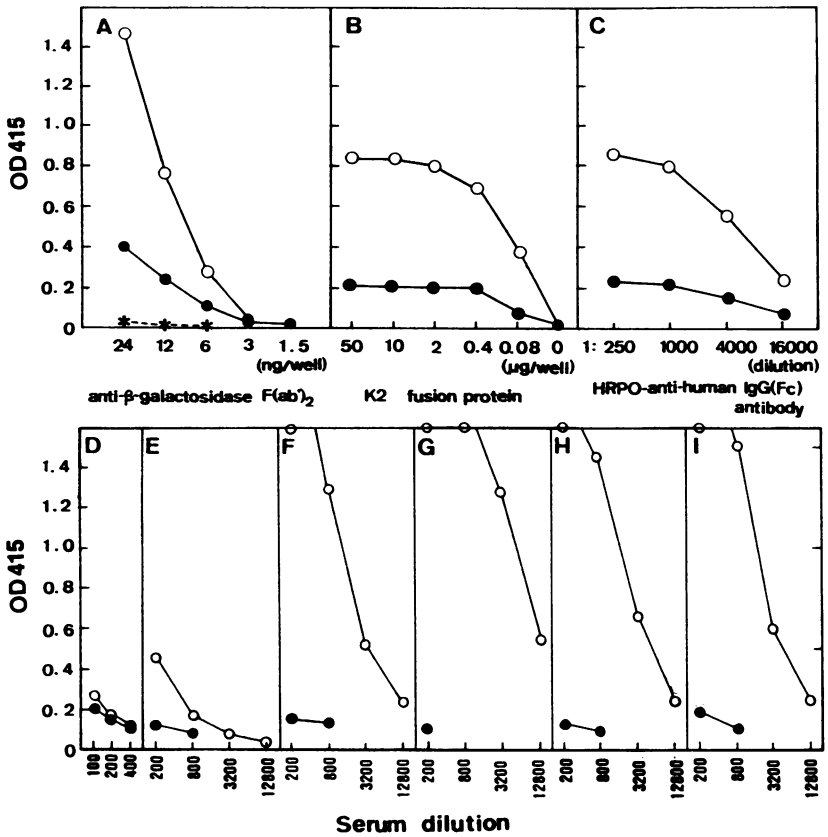

FIG. 2. (A, B, and C) Optimization of the experimental conditions for the sandwich ELISA. Each sequential step in the reaction was done as follows. (i) Each well of a 96-well plate was coated with 12 ng of anti-β-galactosidase IgG F(ab')$_2$ fraction. (ii) Bacterial extract (2 μg) containing the K2(EBNA-1) fusion protein was added. (iii) A human serum sample, TWM1, diluted 1:5,000 (○) or 1:25,000 (●) was added. (iv) Bound human antibodies were detected with HRPO-conjugated anti-human IgG (Fc-specific) antibody. Dose dependence in each step was examined by changing the amount of the reagents as follows: (A) the coating anti-β-galactosidase IgG F(ab')$_2$ fraction; (B) the bacterial extract containing the K2 fusion protein; (C) HRPO-conjugated anti-human IgG (Fc-specific) antibody. The broken line with asterisks in panel A shows the reaction without the fusion protein and human serum. (D through I) Titration curves of antibodies to K2 (○) or β-galactosidase (●) in serum samples from the following individuals: D, an anti-VCA antibody-negative healthy infant (OKA582); E, F, and G, anti-VCA antibody-positive healthy adults (TWM122, TWM125, and TWM21, respectively); H, a patient with Sjögren's syndrome (TWM75); I, a patient with Sjögren's syndrome and systemic lupus erythematosus (TWM96).

sandwich ELISA gave a very good correlation (Fig. 3C), and the correlation coefficient was 0.97.

We comparatively analyzed IgG antibody titers against EBNA by the ACIF test with Raji cells and the EBNA-1 domain by the ELISAs with the fusion proteins (Fig. 4). Anti-VCA antibody-positive serum samples from healthy individuals (solid circles) and from patients clinically diagnosed as having IM (open circles) were examined. The correlation coefficient between the ACIF test and the indirect ELISA with SII was 0.89 (Fig. 4A), although the OD of the serum samples with ACIF titers of 160 or 320 appeared to be saturated. The correlation coefficient between the ACIF test and the sandwich ELISA with K2 was 0.92 (Fig. 4B). These results indicate that the antibody titers determined by the two ELISA methods are in good agreement in most cases with those determined by the conventional ACIF analysis. It should be noted, however, that both the indirect and the sandwich ELISAs detected significant anti-EBNA-1 domain antibody titers in samples TWM37 and TWM128, which are marked by arrows in Fig. 4, which had titers below the threshold titer (1:10) in the ACIF test with Raji cells. The presence of anti-EBNA-1 antibodies in the two serum samples was also shown by immunoblotting analysis at a serum dilution of 1:10 with the lysate of Raji cells or the K2 fusion protein as the antigen (data not shown), indicating that the reactivity with K2 detected by the ELISAs was specific to the EBNA-1 domain.

DISCUSSION

The conventional ACIF test established by Reedman and Klein (23) and Henle et al. (10) has been used widely and routinely for the purpose of research and clinical diagnosis. Recently, separate measurement of antibodies to EBNA-1 and to EBNA-2 by the ACIF with EBNA-1- and EBNA-2-expressing eukaryotic cells obtained by gene transfection suggested that anti-EBNA antibodies detected by the conventional ACIF test consist mainly of anti-EBNA-1 antibodies (9, 27). In this work, we established an indirect ELISA and a sandwich ELISA to titrate antibodies against EBNA-1 with chimeric EBNA-1 fusion proteins and compared the titers of anti-EBNA antibodies determined by the conventional ACIF test with those of antibodies to the EBNA-1 domain by the ELISAs. The ELISAs in this work detected antibodies specific to the carboxyl domain of EBNA-1 (Fig. 1 and 3A). Although the fusion proteins have fewer epitopes

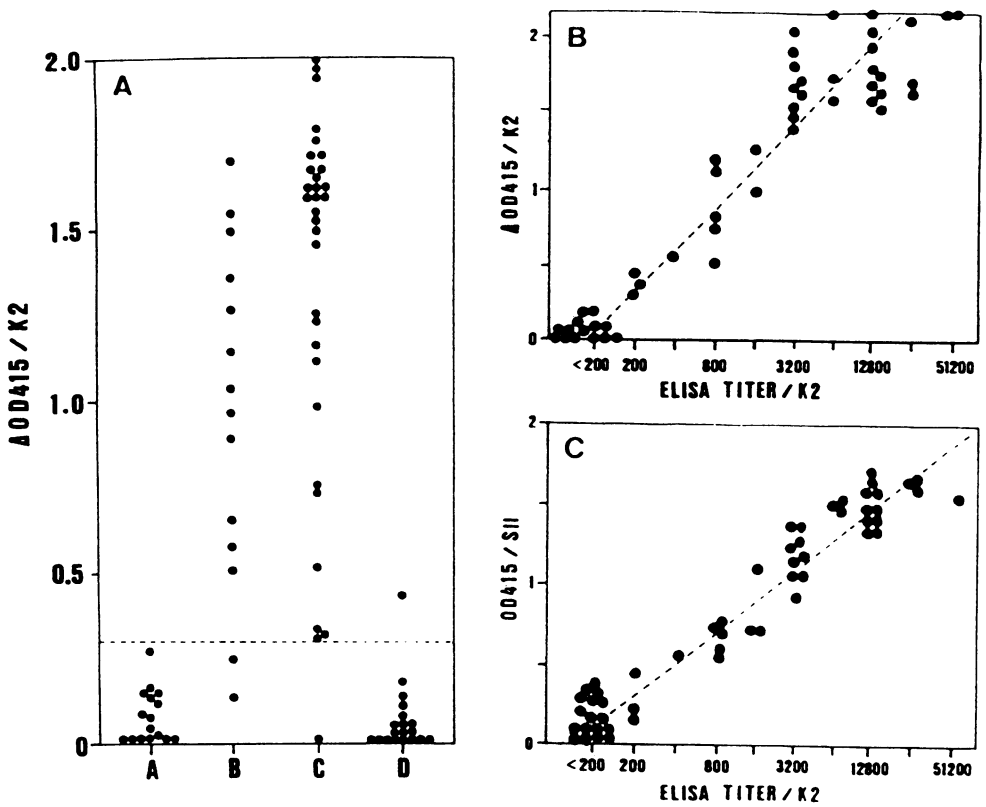

FIG. 3. (A) Sandwich ELISA with the K2 fusion protein. Anti-K2 antibody titers in serum samples from the following groups were measured in terms of ΔOD_{415} values at a serum dilution of 1:200: A, 16 anti-VCA antibody-negative healthy individuals; B, 16 anti-VCA antibody-positive healthy infants from 1 to 3 years old; C, 38 anti-VCA antibody-positive healthy adults; D, 19 IM patients. Serum samples which had antibody titers below 0.3 ΔOD_{415} are shown below the broken line. (B) Correlation of the two measurements by the sandwich ELISA with K2. ΔOD_{415} values at a serum dilution of 1:200 (the y axis) and ELISA titers (the x axis). Serum samples from groups C and D were used. (C) Correlation of the OD_{415} values obtained by the indirect ELISA with SII (the y axis) and the titers obtained by the sandwich ELISA with K2 (the x axis).

than the intact molecules, the titers of anti-EBNA-1 antibodies obtained by the ELISA methods were in a very good agreement with those determined by the conventional ACIF test (Fig. 4A and B). There was no big discrepancy in the antibody titer between the ELISAs and the ACIF test in the titer range above 10 in the ACIF (Fig. 4). The observed distribution of anti-EBNA-1 domain IgG antibody titers suggests that the ELISA methods are useful in serodiagnosis.

The anti-EBV antibody patterns in acute IM are characterized by raised antibody titers to VCA and a usually undetectable titer of antibodies against EBNA (10). Niederman and Miller (22) reported that antibodies to EBNA-1 arose long after anti-EBNA had become detectable by the ACIF test. In addition, Henle et al. (13) indicated, by ACIF, that an increase in anti-EBNA-2 antibody titer preceded one in anti-EBNA-1 antibody titer in IM patients and that most serum samples obtained within 3 months after onset of the disease contained little anti-EBNA-1 antibody. In this study, we examined, by ELISA, anti-EBNA-1 titers of serum samples obtained from 19 IM patients within 2 months after onset of the disease and observed little or no antibody against EBNA-1 in these IM patients (Fig. 1 and 3).

Thus far, attempts to use ELISA for the titration of antibodies against EBNA-1 with the following antigens were reported: EBNA-1 purified from an EBV-positive Burkitt's cell line (19); the purified 28K-EBNA-1 synthesized in *E. coli* (21); and oligopeptides which were synthesized according to the deduced protein sequence of EBNA-1 (4, 18, 24). Our results with the indirect ELISA of EBNA-1 (Fig. 4) were essentially consistent with the result by Milman et al. (21). On the other hand, several reports (4, 18, 26) suggested that the major EBNA-1 epitope was not in the carboxyl domain but in the glycine-alanine repeat of EBNA-1, which is not included in the fusion protein used in this report, based on the results obtained with their synthetic oligopeptides. A reason for the reported weak antigenicity of the carboxyl domain of EBNA-1 in their experiments may be the limited length of their oligopeptides. We reported that the carboxyl domain of EBNA-1 contains a hypothetical helix-loop-helix structure, which is a characteristic structure common to a family of DNA-binding proteins (14), and we believe that their oligopeptides could not form the higher-order structure which may constitute a portion of EBNA-1's major epitopes. Further comparative study by the ELISA with an EBNA-1 fusion protein(s) containing the glycine-alanine repeat will clarify the relative antigenicity of epitopes in the different domains.

The indirect ELISA with the SII fusion protein and the sandwich ELISA with the K2 fusion protein in this work showed essentially the same specificity and sensitivity (Fig. 3C and 4). The sandwich ELISA with SII gave the same titers as that with K2 (data not shown). The indirect ELISA is more convenient than the sandwich ELISA, but the

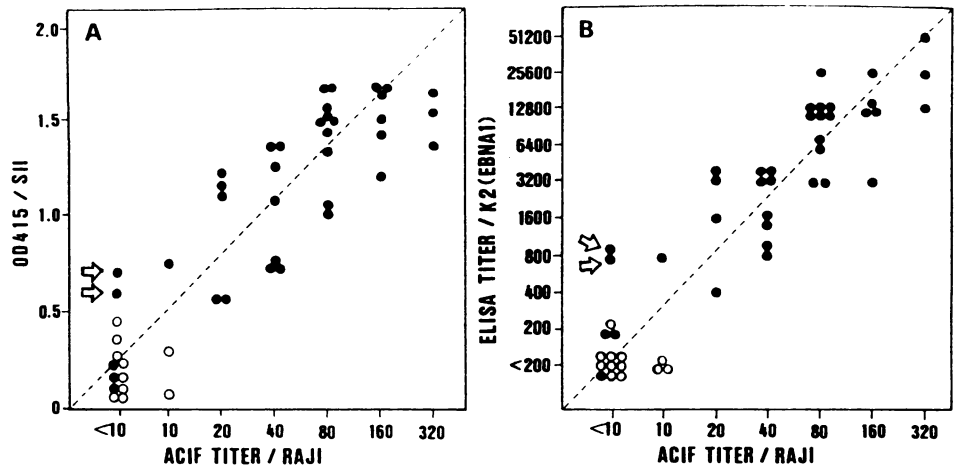

FIG. 4. (A) Correlation between the OD_{415} values obtained by indirect ELISA with SII (the y axis) and ACIF with Raji cells (the x axis). Serum samples from 38 healthy individuals (●) and 10 IM patients (○) were analyzed. (B) Correlation of the titers to K2 determined by the sandwich ELISA (the y axis) and by ACIF with Raji cells (the x axis). Serum samples from 38 healthy individuals (●) and 12 IM patients (○) were analyzed. The two serum samples marked by arrows are discussed in the text.

former requires highly purified protein and preabsorption. We used SII instead of K2 to obtain a large amount of the fusion protein, although SII lacks the carboxyl-terminal 23 amino acids of K2. On the other hand, a so-called sandwich assay (5), in which specific antibody is used to capture and fix specific antigens onto the solid phase at the first step, is known to be useful when it is difficult or costly to isolate specific antigens. The sandwich assay, however, requires a highly specific antiserum or monoclonal antibody to the antigen of interest. This study demonstrated that the use of fusion proteins for a sandwich assay can overcome the necessity for the use of such antiserum or antibody. Furthermore, the sandwich ELISA with chimeric fusion protein is theoretically applicable to titration of antibodies against EBNA-2, -3, -4, and -6, because we have already constructed and characterized more than 20 DNA constructs which expressed β-galactosidase fusion proteins containing the EBNA constituents (15). Then, we measured antibody titers against the EBNA-2, -3, and -4 fusion proteins individually by the same procedure as used for anti-EBNA-1 antibody (data not shown). It is, however, difficult at the moment to strictly evaluate the data obtained by the ELISA for IgG to these EBNAs, because there is no standard method with which they can be compared. Establishment of eukaryotic cell lines which express each of the EBNA polypeptides and comparison of the results with the ELISAs and the ACIF with such cell lines will clarify the specificity and sensitivity of the ELISAs.

Finally, it should be noted that the investigation of possible association of EBV infection with some autoimmune diseases through ACIF or indirect fluorescent-antibody tests has been hampered by the presence of autoantibodies, especially antinuclear antibodies, in the patients' serum samples. We showed that antibodies specific to the EBNA-1 domain were detectable quantitatively in the serum samples from patients with autoimmune diseases by the ELISA developed in this study (Fig. 2H and I). Although it was reported that the glycine-alanine repeat in EBNA-1 contained the epitopes that cross-reacted with a cellular protein(s), the glycine-alanine repeat is not included in the K2 and SII fusion proteins (8). In any event, the ELISA methods will be of help in studying the association of EBV infection with some autoimmune diseases. These rapid and convenient ELISAs can contribute to seroepidemiologic studies and widely practiced clinical analyses of antibody responses to EBNAs in IM and other diseases.

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REFERENCES

1. **Berkel, A. I., W. Henle, G. Henle, G. Klein, F. Ersoy, and O. Sanal.** 1979. Epstein-Barr virus-related antibody patterns in ataxia telangiectasia. Clin. Exp. Immunol. **35**:196–201.
2. **de-The, G.** 1982. Epidemiology of Epstein-Barr virus and associated diseases in man, p. 25–103. *In* B. Roizman (ed.), The herpesviruses, vol. 1. Plenum Publishing Corp., New York.
3. **Dillner, J., and B. Kallin.** 1988. The Epstein-Barr virus proteins. Adv. Cancer Res. **50**:95–158.
4. **Dillner, J., L. Sternås, B. Kallin, H. Alexander, B. Ehlin-Henriksson, H. Jorvall, G. Klein, and R. Lerner.** 1984. Antibodies against a synthetic peptide identify the Epstein-Barr virus-determined nuclear antigen. Proc. Natl. Acad. Sci. USA **81**: 4652–4656.
5. **Engvall, E.** 1980. Enzyme immunoassay ELISA and EMIT. Methods Enzymol. **70**:419–439.
6. **Epstein, M. A., and B. G. Achong.** 1979. Introduction: discovery and general biology of the virus, p. 1–22. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, Berlin.
7. **Evans, A. S.** 1978. Infectious mononucleosis and related syndromes. Am. J. Med. Sci. **276**:325–339.
8. **Fox, R., R. Sportsman, G. Rhodes, J. Luka, G. Pearson, and J. Vaughan.** 1986. Rheumatoid arthritis synovial membrane contains a 62,000-molecular weight protein that shares an antigenic epitope with the Epstein-Barr virus-encoded associated nuclear antigen. J. Clin. Invest. **77**:1539–1547.
9. **Hearing, J. C., J.-C. Nicolas, and A. J. Levine.** 1984. Identification of Epstein-Barr virus sequences that encode a nuclear antigen expressed in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **81**:4373–4377.
10. **Henle, W., G. Henle, and C. A. Horwitz.** 1974. Epstein-Barr virus-specific diagnostic tests in infectious mononucleosis. Hum. Pathol. **5**:551–565.
11. **Henle, W., and G. Henle.** 1979. Seroepidemiology of the virus,

p. 61–78. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, Berlin.
12. **Henle, W., and G. Henle.** 1985. Epstein-Barr virus and human malignancies, p. 201–238. *In* G. Klein (ed.), Advances in viral oncology, vol. 5. Raven Press, New York.
13. **Henle, W., G. Henle, J. Andersson, I. Ernberg, G. Klein, C. Horwitz, G. Marklund, L. Rymo, C. Wellinder, and S. E. Straus.** 1987. Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. Proc. Natl. Acad. Sci. USA **84:** 570–574.
14. **Inoue, N., S. Harada, T. Honma, T. Kitamura, and K. Yanagi.** 1991. The domain of Epstein-Barr virus nuclear antigen 1 essential for binding to *oriP* region has a sequence fitted for the hypothetical basic-helix-loop-helix structure. Virology **82:**84–93.
15. **Inoue, N., S. Harada, and K. Yanagi.** 1991. Synthesis of fusion proteins of Epstein-Barr virus nuclear antigens in *E. coli* and their antigenicity. J. Virol. Methods **31:**301–314.
16. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1919. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology, 2nd ed. Raven Press, New York.
17. **Kino, T. P., Y. Li, and L. Kochoumian.** 1978. Preparation of protein-conjugates *via* intermolecular disulfide bond formation. Biochemistry **17:**1499–1506.
18. **Linde, A., B. Kallin, J. Dillner, J. Andersson, L. Jagdahl, A. Lindvall, and B. Wahren.** 1990. Evaluation of enzyme-linked immunosorbent assays with two synthetic peptides of Epstein-Barr virus for diagnosis of infectious mononucleosis. J. Infect. Dis. **161:**903–909.
19. **Luka, J., R. C. Chase, and G. R. Pearson.** 1984. A sensitive enzyme-linked immunosorbent assay (ELISA) against the major EBV-associated antigens. I. Correlation between ELISA and immunofluorescence titers using purified antigens. J. Immunol. Methods **67:**145–156.
20. **Masucci, M. G., R. S. Szigeti, I. Ernberg, G. Masucci, G. Klein, J. Chessels, C. Sieff, S. Lie, A. Glomstein, L. Businco, W. Henle, G. Pearson, K. Sakamoto, and D. T. Purtillo.** 1981. Cellular immune defects to Epstein-Barr virus-determined antigens in young males. Cancer Res. **41:**4284–4291.
21. **Milman, G., A. L. Scott, M.-S. Cho, S. C. Hartman, D. K. Ades, G. S. Hayward, P.-F. Ki, J. T. August, and S. D. Hayward.** 1985. Carboxyl-terminal domain of the Epstein-Barr virus nuclear antigen is highly immunogenic in man. Proc. Natl. Acad. Sci. USA **82:**6300–6304.
22. **Niederman, J. C., and G. Miller.** 1986. Kinetics of the antibody response to *Bam*HI-K nuclear antigen in uncomplicated infectious mononucleosis. J. Infect. Dis. **154:**346–349.
23. **Reedman, B. M., and G. Klein.** 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer **11:**499–520.
24. **Rhodes, G., D. A. Carson, J. Valbracht, R. Houghten, and J. H. Vaughan.** 1985. Human immune responses to synthetic peptides from the Epstein-Barr nuclear antigen. J. Immunol. **134:**211–216.
25. **Ricksten, A., B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Rymo.** 1988. The *Bam*HI E region of the Epstein-Barr virus genome encodes three transformation-associated nuclear proteins. Proc. Natl. Acad. Sci. USA **85:** 995–999.
26. **Rumpold, H., G. H. Rhodes, P. L. Bloch, D. A. Carson, and J. H. Vaughan.** 1985. The glycine-alanine repeating region is the major epitope of the Epstein-Barr nuclear antigen-1 (EBNA-1). J. Immunol. **138:**593–599.
27. **Seigneurin, J. M., M. F. Lavoue, O. Genoulaz, G. W. Bornkamm, and G. M. Lenoir.** 1987. Antibody response against the Epstein-Barr virus-encoded nuclear antigen 2 (EBNA2) in different groups of individuals. Int. J. Cancer **40:**349–353.
28. **Vilmer, E., G. M. Lenoir, J. L. Virelizier, and C. Griscelli.** 1984. Epstein-Barr serology in immunodeficiencies: an attempt to correlate with immune abnormalities in Wiskott-Aldrich and Chediak-Higashi syndromes and ataxia telangiectasia. Clin. Exp. Immunol. **55:**249–256.